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THE FEEDING HABIT OF TERMITE CASTES AND ITS RELATION TO THEIR INTESTINAL FLAGELLATES.

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More than 100 genera and approximately 1,200 species of termites are known; and each species is usually composed of five castes, with male and female individuals in each. Three of these castes, commonly referred to now as *first, second*, and *third forms*, are responsible for the reproduction of other individuals like themselves and for two other castes, *workers* and *soldiers*, which, although they possess reproductive organs, have given up the reproductive function (if they ever possessed it).

Nearly all the observations and experiments in the present paper have been carried out on one of the most common North American termites, *Reticulitermes flavipes* Kollar, whose castes (Thompson and Synder, '20) may be briefly described as follows:

(1) *First form*, which has three well-defined phases of development: (*a*) the nymphs (Figs. 1, 2), with long wing pads, creamy white body 1.3–1.4 mm. long, light brown eyes; (*b*) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (*c*) the older males and females (Fig. 3), with enlarged abdomens and the scales of the shed wings, body 7–14 mm. long.

(2) *Second form*, which, like the first form, has three well-defined phases of development: (*a*) nymphs (Figs. 4, 5), with short wing pads and colorless body and eyes; (*b*) the young adults, with short scaly wing vestiges, straw-colored or grayish body 6–7 mm. long; (*c*) the older adults (Fig. 6), with wing vestiges, enlarged abdomen, body length 7–12 mm.

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(3) *Third form*, which also has three developmental phases: (*a*) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (*b*) the young adults, no wing vestiges, head and body white, 7–9 mm. long.

(4) The *worker* (Fig. 7), wingless, with grayish abdomen, only two developmental phases, *i.e.*, nymphs and adults, salivary glands small and very little fatty tissue is present in the body, blind.

(5) The *soldier* (Figs. 8, 9), wingless, with elongated head covered with thick yellowish chitin, mandibles very large, long, dark brown, slender and curved, abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth as in reproductive castes.

Thompson ('17) showed that the newly hatched nymphs of *Reticulitermes flavipes*, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (*a*) reproductive nymphs, from which the three fertile adult castes develop, and (*b*) the worker-soldier nymphs, from which the sterile adult castes develop. By the time reproductive nymphs had attained a body length of 1.3–1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated internally by the time they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

These five castes occur in most termites. Known exceptions are: the third form occurs in few, if any, species of the family Termitidae; a true worker caste is not present in the genera *Termopsis* Heer and *Neotermes* Holmgren, but a large-headed worker-like reproductive form is present; two genera, *Kalotermes* Hagen and *Cryptotermes* Banks, have no worker caste; the genus *Anoplotermes* F. Müller has no soldier caste; some species have as many as three types of soldiers, which, if counted as castes, make these species have seven castes, provided the five described above are all present.

Grassi ('93) calls the first forms "true" or "perfect" insects,

or "royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms, he thinks, are always ready to take the place of the royal forms in case of need. This author believes the castes are a product of environment and special feeding; what environment and special feeding, he does not consider. Bugnion ('12, '13) and Imms ('19) believe the castes are a product of the germplasm. The field observations that have been made (Snyder, '15, '16) support this contention. So do the morphological studies of Thompson ('17). But we really *know* absolutely nothing about what produces the castes. The question is badly in need of study. Jucci ('20),[1] who recently announced the discovery of a particular diet which brings about caste production, has added nothing but confusion, subtlely clothed in high-sounding phraseology, to the origin of a most interesting phenomenon.

From the description that was given of the castes, it may be clearly seen that very great morphological differences exist within a termite species, *i.e.*, the castes are quite distinct. It also seems likely that physiologically the castes are equally distinct.

The writer ('23*b*, '24*a*, '25*b*) has definitely shown that the removal of the intestinal protozoa from at least two genera of termites (*Reticulitermes* and *Termopsis*) makes it impossible for them to live on their normal diet of wood. He ('23*a*) has also shown that if termites harbor protozoa, they must feed on wood or cellulose. In the present paper a study of the various castes has been made in order to determine whether or not what is true for termites in general is also true for each caste throughout its life-cycle. In addition to this, some data on the physiological differentiation of castes and the relation of the castes to each other have been obtained.

Experiments and Observations.

Forty to fifty colonies of *Reticulitermes flavipes* were collected and have been kept for the past three years in the laboratory where they have been carefully observed. More than 300 second

[1] After this paper had gone to press a voluminous monograph by Jucci appeared, in which much attention is given to considerations of minor importance. A good beginning has been made, but the problem of the origin of termite castes has not been solved.

and third form young adults and 150 soldiers have been isolated from these colonies and have been used in experiments. And several hundred have been kept for observations. Thousands of workers and first forms have figured in experiments and observations.

One experiment, which was later duplicated five times with the same result each time, was carried out as follows: Five individuals of each of the five castes, workers, soldiers, third forms, second forms, and first forms, were placed in each of five large size sputum jars with food. These jars were kept in a moist chamber, so that the moisture, as well as temperature and light, would be identical in all the experiments. Experience has also shown that this is the best way to keep a colony of termites normal in the laboratory. Another experiment, which was duplicated three times with the same result each time, was carried out in the same manner as the experiment just mentioned except that the termites were kept in screw-top jars which were not placed in a moist chamber. In both of these experiments the second forms, the third forms, and the soldiers were all dead, in every instance, within three to four weeks, while the first forms and workers were able to live indefinitely.

Many observations were made daily throughout the course of these experiments and no castes except the workers and first forms were ever seen to take food. At various intervals several individuals were killed and their intestinal contents were carefully examined microscopically, with the result that no wood particles were ever present except in the workers, first forms, and soldiers—just how the soldiers came to have wood particles in their intestines and yet were not able to live will be made clear later. It seems quite evident, indeed, then, that the young second and third form adults do not feed on wood; that they get their nourishment perhaps in the form of salivary secretions from the xylophagous members of the colony.

Why do some castes die when placed by themselves? Is it because they do not take food? To test this point further, twenty experiments were carried out, ten where one worker was placed in a vial with two second form individuals and ten where one worker was placed in a vial with two soldiers. Under this condition, the second forms and soldiers were able to live in-

definitely. Thus one worker can at least support itself and two soldiers or two second forms. How much more a worker can do, was not determined.

Why do the second and third form young adults not take food when placed by themselves? A very large number of individuals of the second and third form castes were examined carefully for protozoa and in no instance were protozoa found after the final molt. In this connection it is interesting to note that every time any individual of any cast molts, its intestinal protozoa are lost, but in all molts, except the last one of the second and third forms, the protozoa are regained very quickly. Sometimes—though very rarely indeed—as the intestine slips off during the molting process, a portion of it is eaten before the protozoa die; but, as a rule, if an individual at the time of molting is placed to itself, it does not regain its intestinal protozoa and, as a consequence, dies within three weeks or thereabouts. But the first form regains its intestinal protozoa after the final molt. Why is it able to do this, while the second and third forms cannot? Obviously, one reason why the second and third forms cannot live by themselves after the final molt is because they have lost their protozoa. But this does not explain why they do not take food, for workers continue to feed after the protozoa have been removed from them experimentally.

Why and how are the protozoa lost? In an effort to throw light on this question, the second form was studied during the short period just before the final molt in which it can be distinguished externally from the first form. It was found that the protozoa gradually disappear during this period and that few, if any, are present at the time of the final molt. It must be noticed that there is also a dimunition in the protozoa in the first form during the period preceding the final molt, but not so great as in the second form, and the protozoa never disappear entirely. This progressive disappearance of the protozoa of the reproductive castes at this time is perhaps brought about by one or both of two things; namely, the salivary secretion which is taken during this period of rapid change and development destroys the protozoa and, as a consequence, wood-feeding must be given up; or, so much salivary secretion is taken that wood-feeding is thus made unnecessary and is, therefore, given up,

in which case the protozoa die due to wood starvation, just as happens experimentally (Cleveland, '25*b*) when any normal wood-feeding termite containing a large number of protozoa is starved. In all three reproductive castes it is quite evident that much less wood is eaten at this time; but in the first form the wood diet is not entirely given up; it may be curbed greatly, though never supplanted, by the salivary diet. It is interesting in this connection to note that the first forms eat much more wood shortly after the final molt and, because of this, the protozoa increase rapidly in number. They certainly receive no salivary secretion from workers for sometime if they leave (swarm) the parent colony to start a new colony, and nearly every one, if not every one, leaves or is killed. Their only food is wood until they rear workers to furnish them salivary secretions again, and when this is done, they again progressively cease to eat wood, finally giving up the habit entirely, at which time they lose all their protozoa and become dependent on the xylophagous members of the colony for the rest of their lives. It is interesting here to note that instinctively this dependence, which is perhaps inevitable, is well taken care of or looked forward to, because mostly workers are reared in the first brood of such a reproductive pair. But would they become dependent if not allowed to rear workers, that is if the larvæ were killed or taken from them?

We have already said that one reason why the second and third form young adults die when placed by themselves is because they lose their protozoa, and the protozoa are lost because these forms do not feed on wood, the second and perhaps more fundamental reason why death results when such individuals are isolated. But why do they not eat wood? According to Thompson and Snyder ('20) the jaw muscles and many others, particularly those in the head, of the first form degenerate during the post-adult stage. "This degeneration of the jaw muscles," they state, "is due to the fact that the reproductive forms are now fed by the workers on partly digested food and no longer masticate wood as they were compelled to do before the first broods of workers were raised." These authors also observed that the jaw muscles in the second and third forms in the post-adult stage had degenerated, though they did not state when the degeneration occurred. If, in the first forms, it occurs, as they

state, when workers supply them with "partly digested food," thus making the eating of wood unnecessary, may we not reasonably assume that in the second and third forms it also occurs when partly digested food is supplied them by workers and wood-feeding is permanently given up, that is at the time of the final molt or thereabouts, considerably earlier than in the first forms. If this is true, we know why the second and third form young adults when experimently placed by themselves do not eat wood and die; their jaw muscles have degenerated, thus making it impossible for them to eat wood, and they can only survive when fed by workers.

But what causes the jaw muscles to degenerate? Thomson and Snyder ('20) think it is due to disuse, brought about when the salivary diet takes the place of the wood diet. If this is true, then, the second and third forms must get more salivary secretion or partly digested food than the first form, since they lose the ability to eat wood and, of course, their protozoa that digest the wood for them, much earlier in life, at least two years earlier. If it is true that salivary secretion brings about a degeneration of the jaw muscles, why are the reproductive forms fed a salivary diet? Certainly not to make the jaw muscles degenerate, for this is surely only a consequence of some deeper, underlying reason for feeding a salivary diet to the reproductive forms. In other words, if the jaw muscles do not degenerate except through disuse, the salivary secretion is perhaps a necessity and may play a vital part either in accelerating or changing the course of development. On the other hand, if the jaw muscles degenerate in the absence of a salivary diet, that is not because of disuse, it may be that the function of such a diet is simply to take the place of a wood diet which becomes impossible. If this is true, the question, why do the jaw muscles degenerate, is perhaps as vital as caste production itself, which, if a result of food, would perhaps be stopped, or at least held in abeyance to some extent, if individuals were isolated very early. A few attempts have been made to get second forms early in the gradual decline of wood-feeding, which occurs simultaneously with a progressive dimunition in the number of protozoa. It was found that such second form individuals when isolated early can live by themselves longer than if allowed to remain with

workers for a while and isolated at a later stage of development, thus showing that at the time of the earliest isolation they had already begun to gradually lose the ability to maintain themselves and that they lose it more quickly when they remain with workers who fed them. Thus the decline in wood-feeding occurs anyway regardless of the time of the isolation, although more slowly seemingly when workers are not present. However, this experiment does not mean much, for long before the external differentiation has occurred which makes the distinction between the second and first form possible, several quite noticeably distinct internal microscopic differentiations had already occurred, thus showing that the differentiation cause had its origin much earlier and had been operating for sometime before the attempt to arrest it was made. Perhaps the workers could distinguish them and had been feeding them. To settle the question in this way, one should begin the isolation at an earlier stage. But these microscopic differences just referred to are only distinguishable in *Reticulitermes* after fixation and staining. Possibly in other genera the task will be less difficult, and we may be able to determine definitely what effect, if any, a salivary diet has on caste production, whether the decline in wood-feeding is caused by the salivary diet or whether the salivary diet has to be substituted for the wood diet after the jaw muscles have degenerated.

Since the second and third form young adults cannot live by themselves, it, of course, follows that they cannot start new colonies in the absence of workers. Incidently, the fact that they do not harbor protozoa shows beyond question, regardless of the fact that they cannot eat wood, that they never start new colonies in the absence of workers. This would be true just the same even if the protozoa were not absolutely necessary to their existence, because if new colonies were started by second and third forms, these colonies would not have protozoa in any of their castes. No such colonies have ever been found; consequently workers must be present when these forms head a colony.

It should be mentioned here that in the genus *Termopsis*, which has no true worker caste, it appears from the observations that have been made that the ability to eat wood is not lost in the second and third forms. But the observations on this genus are

really too few to warrant a conclusion further than that these forms certainly do not lose their protozoa and the ability to eat wood as early as they do in *Reticulitermes*, which has workers. If the degeneration of the jaw muscles occurs in the reproductive forms when and because workers supply them with a salivary or partly digested diet, we should not expect to find degenerate jaw muscles and enlarged dependent reproductive forms in those genera where workers are not present, unless, of course, the young nymphs play the same rôle that workers do in other genera.

We will now return to the soldier caste and take up the question, why are adult soldiers unable to live by themselves? Simply because their very large and heavily chitinized mandibles (Fig. 9) will not permit them to eat wood. They, too, like the second and third forms, lose the ability to eat wood but from a growth process rather than one of degeneration. Before their mandibles grew so large, they could eat wood and could live by themselves. Could these mandibles be altered by a change in diet? Did a diet produce them? We cannot answer either question.

Soldiers can digest wood, for they have protozoa in their intestines to do it for them, but are unable to eat it. They lose the ability to eat wood without losing their protozoa, for they get protozoa, just as all other castes do, very early in life from the ani of the xylophagous members of the colony. As we have said, owing to their very enormous and highly specialized mandibles (Fig. 9), they cannot chew wood, but they manage to ingest proctodael wood particles—partially digested perhaps—which have passed through the alimentary canal of those members in the colony capable of chewing wood. Their intestines are considerably smaller and they harbor a smaller quantity of protozoa than workers of the same size. Soldiers, then, are not as dependent, in one sense, on workers or permanent wood-chewing members of the colony as the second and third forms are, for they do not require totally predigested food, such as the salivary secretions upon which the second and third forms probably feed entirely after the final molt.

Summary.

All results were obtained from laboratory colonies which have been carefully studied during the past three years. Many of these results have been verified by field observations.

At every stage in the life-cycle of any caste where wood is eaten, protozoa are present. When wood is not eaten or obtained in some way, protozoa are never present.

Second and third form young adults have lost the ability to eat wood. The protozoa in these castes disappear concomitantly with the loss of the ability of their host to feed on wood, and by the time the wood-eating ability is lost, they have all disappeared. This occurs about the time of the final molt and is perhaps brought about by the feeding of salivary secretions which take the place of the wood diet. In all castes, the protozoa are lost during molting, but they are soon regained, except in the final molt of the second and third forms, in which forms they are never regained because, owing perhaps to the degeneration of their jaw muscles, these forms have lost the ability to eat wood. What causes the jaw muscles to degenerate is not definitely known. It may be inherent in these castes, as much a part of them as anything else. If it is, then the salivary-feeding is hereby made necessary and takes the place of the wood diet when the jaw muscles degenerate. But a more plausible possibility is that these forms are fed so much salivary secretion that they cease to feed on wood and because of this their jaw muscles degenerate through disuse, and thus the ability to feed on wood is lost forever.

The first form and the worker always eat wood, except in the post-adult stage of the life-cycle of the first form, where it, too, after having attained an old age loses the ability to eat wood and becomes dependent on the workers and young undifferentiated nymphs (when present) which it has reared. It is noteworthy that mostly workers are raised in the first brood.

Adult soldiers, owing to their large mandibles, cannot eat wood (cannot chew it), though they obtain it, together with protozoa from the ani of the xylophagous members of the colony. Soldiers, like workers, harbor protozoa throughout their life-cycle. Young soldiers (soldier nymphs), before they obtain the

large mandibles, can chew wood for themselves. So can the second and third forms, during early life.

A caste which cannot eat wood, or, thinking in terms of the protozoa, a caste which does not harbor protozoa, cannot live by itself. Such individuals are dependent on the wood-eating members of the colony for support; consequently adults of the second form, third form, and soldier castes must be supported by other members of the colony. But the soldiers, in one sense, are not as difficult to support as the second and third forms, since they can digest for themselves the partially digested woody material which has passed through the alimentary canal of the xylophagous members of the colony before they receive it; while the second and third forms, since they feed exclusively on the salivary secretions, must subsist entirely on predigested food.

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Explanation of Plate I.

Figs. 1–9. The Castes of *Reticulitermes flavipes.*

Fig. 1. Nymph of first form, with long wing pads, creamy white body from 1.3–1.4 mm. long, light brown eyes.

Fig. 2. Side view of Fig. 1.

Fig. 3. Post adult first form queen, with enlarged abdomen, the scales of the shed wings, body length varies from 7–14 mm. This figure × 5.

Fig. 4. Nymph of second form, with short wing pads which never develop into wings, colorless body and eyes.

Fig. 5. Side view of Fig. 4.

Fig. 6. Post adult of second form queen, with wing vestiges, enlarged abdomen, body length varies from 7–12 mm. This figure × 5.5.

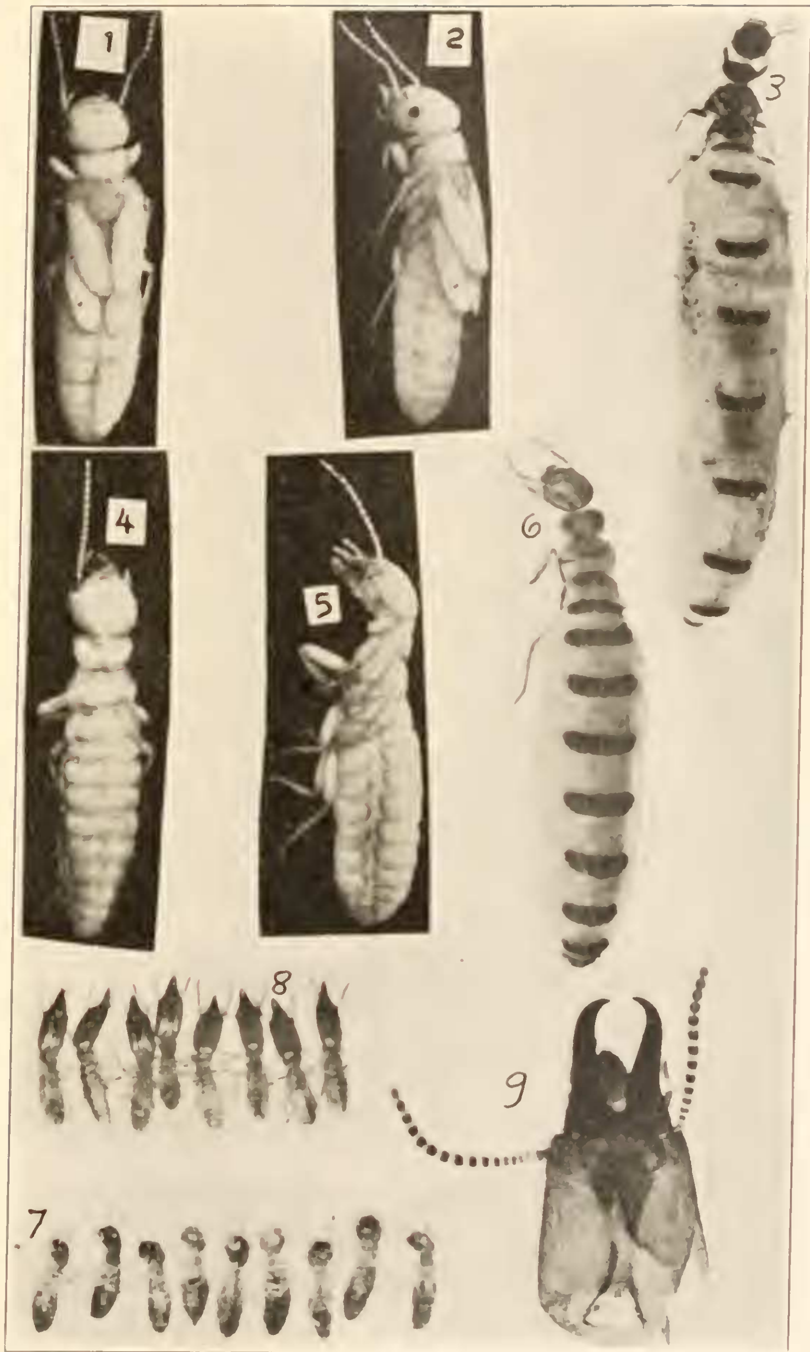
Note: The third form caste which for lack of space is not shown, has no wing vestiges, eyes cannot be seen except in stained material, is smaller having a body in the post adult stage of 7–9 mm.

Fig. 7. A group of workers enlarged three times.

Fig. 8. A group of soldiers enlarged three times.

Fig. 9. Mandibles of adult soldier which make the eating of wood impossible. × 16.

All figures (photographs) after Snyder.



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THE EFFECTS OF OXYGENATION AND STARVATION ON THE SYMBIOSIS BETWEEN THE TERMITE, *TERMOPSIS*, AND ITS INTESTINAL FLAGELLATES.

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INTRODUCTION.

In a previous paper ('24*a*) the writer reached the conclusion that xylophagous and protozoa-harboring termites are not able to live on their normal diet of wood after their intestinal protozoa are removed from them by incubation for 24 hours at 36° C. They die within three to four weeks if given a wood diet. When the protozoa are replaced, the termites concomitantly regain their ability to live indefinitely on a diet of wood or cellulose. Thus, the incubation, which removed the protozoa, did not kill the termites *per se*. Also, when termites from which the protozoa had been removed by incubation, were given a diet of fungus-digested cellulose, they were able to live indefinitely. Therefore, the ability to make use of cellulose (to maintain itself indefinitely on a wood diet an animal must be able to digest

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cellulose) seems to reside in the protozoa of the termites rather than in the termites themselves.

In the present paper the protozoa have been removed from the large Pacific Coast termite, *Termopsis*, by two other methods, namely, starvation, and oxygenation, with the same results in each case as were obtained by incubation; the termites were not injured although the ability to live on wood disappeared simultaneously with the loss of the protozoa.

In addition to this, the relation of each species of protozoa to its host and to its neighbors or fellow protozoa, has been worked out by employing the various protozoal-removing methods separately and by combining them. Differential defaunation or the removal of some species of protozoa without affecting the others was thus attained.

Termopsis and its Protozoa.

Three species of *Termopsis* are known. Two of them, *T. angusticollis* Hagen and *T. nevadensis* Hagen, can be distinguished only by an examination of their winged adults. In many of the colonies studied, no winged adults have appeared; consequently it has been impossible to determine whether all the experiments were carried out on one or both of these species. The winged adults which appeared in two colonies were *T. nevadensis*. Both species occur in the same localities in Oregon, California and elsewhere on the Pacific Coast and probably harbor an identical protozoan fauna.[1] But for these reasons, it seems most feasible to avoid specific determinations in this paper.[1]

The protozoa of *Termopsis* were first described by Kofoid and Swezy ('19) as follows: "In *Termopsis angusticollis* four different species of protozoans are invariably present." These they described as *Trichonympha campanula* (Fig. 1), *Leidyopsis sphaerica* (Fig. 2), *Trichomitus termitidis* (Fig. 3), and *Streblomastix strix* (Fig. 4). They state that "in addition to these there are usually present minute forms of two, sometimes three species of flagellates," which these authors did not study. These same small forms have been observed by the writer, but they are very small indeed, and few in number, and have been considered of no moment in the experiments that have been carried out.

[1] Later observations show that both species were used and that they harbor the same protozoa.

With two[2] exceptions (*Leidyopsis* and *Trichomitus*), no special effort has been made to verify the morphological studies of Kofoid and Swezy. However, as will be seen later, the writer has had occasion to observe many of these protozoa in almost pure cultures, which simplifies the matter of morphological details considerably, for unless one has really seen this overwhelming mass of squirming, wriggling, undulating protozoa which greet the eye when a termite's intestinal content is viewed under the microscope, one cannot form any conception whatever of the immense difficulty envolved in attempting to study in detail any of them except the very large and dominant genus *Trichonympha*. Of course, if one has a suitable medium in which to dilute the intestinal contents, these difficulties of observation are obviated greatly, but Kofoid and Swezy state that they did not find such a medium; hence it was difficult for them to make out the finer structures of the two smaller forms, *Trichomitus* (?) and *Streblomastix*.[2]

Leidyopsis, when millions of individuals are viewed in a suitable medium and unobscured by other protozoa, is not nearly so rounded as Kofoid and Swezy have figures from stained specimens (Fig. 2). This rounding up which they show is an abnormality of fixation. In living material it occurs also unless the observation is made in a suitable medium (Cleveland, '25*d*).

In the *Termopsis* material—most of which came from Oregon, though three colonies were obtained from California—which the writer has studied there is certainly a species of *Trichomonas* present; whether or not *Trichomitus* is also present it seems almost impossible to say, though all evidence indicates that it is not. For instance, in those hosts which were experimentally freed of *Trichonympha* and *Leidyopsis*, thus affording a wonderful opportunity to study *Trichomonas* (and *Trichomitus* too if present), an opportunity which Kofoid and Swezy did not have, an axostyle (the distinction between *Trichomonas* and *Trichomitus*, a genus which Swezy founded in 1915, lies chiefly if not entirely in the presence of an axostyle in *Trichomonas* and the absence of it in *Trichomitus*) can be seen instantly in 50 per cent. of the individuals exclusive of *Streblomastix;* with more study the number of individuals in which an axostyle is visible increases

[2] *Streblomastix* has only four flagella. See addenda.

to 70–80 per cent.; with still more study after fixation and staining the percentage of individuals in which axostyles may be seen increases to 85–90 per cent., though never reaching 100 100 per cent. In other words, the number of individuals which at first glance would be diagnosed as *Trichomitus*, diminishes concomitantly with scrutiny of observation. Professor Kofoid when notified recently by letter of this finding and of the possibility that the species of *Termopsis* in California (although the protozoa in those colonies I examined were the same as in the Oregon *Termopsis*) might harbor *Trichomitus* while in Oregon they harbor only *Trichomonas*, wrote: "We find both *Trichomonas* and *Trichomitus* in the same hosts in California. We have regarded this *Trichomonas* as possibly the one described by Dogiel although we have not attempted to work it up." Professor Kofoid also kindly sent me slides which show an abundance of *Trichomonas*. However, in the papers of Kofoid and Swezy ('19), as noted by the quotations already given, no mention of the presence of *Trichomonas* appeared. This organism in *Termopsis* does not seem to me to be the same one which Dogiel [1] described as *Tetratrichomonas macrostoma* from *Rhinotermes* sp. of Uganda. The two hosts are widely separated and belong to two families; *Rhinotermes* Hagen belongs to the Rhinotermitidæ, and *Termopsis* Heer to the Kalotermitidæ. The species of *Trichomonas* in *Termopsis* has four anterior flagella (elsewhere the statement was made by the writer ('24*a*) that it had three anterior flagella, but more careful examination shows clearly that some individuals have four anterior flagella, which is perhaps the normal number), axostyle and undulating membrane, which place it definitely in the genus *Trichomonas*. I, therefore, describe it (Figs. 5, 6) as *Trichomonas termopsidis* sp. nov., found certainly in *Termopsis nevadensis* Hagen and probably in *T. angusticollis* Hagen.

This is not the time nor the place for a discussion of the genera *Trichomitus* and *Trichomonas*. If *Trichomitus* is present —though I am inclined somewhat to doubt it—it has behaved the same way as *Trichomonas* in the starvation and oxygenation experiments. But, since it is not certain that *Trichomitus* is present or even exists, no mention of this genus will be made in these experiments.

[1] Dogiel, V.; *Jour. ruse de Zoöl.* (Petrograd), 1.

Experiments and Observations.

a. Starvation.

Ten experiments, employing altogether approximately five hundred termites, were carried out as follows: The termites were removed from wood and carefully freed of all wood particles which they attempted to cling to. Then they were placed in large Petri dishes which were kept in moist chambers. In this way the amount of moisture which previous work had shown most desirable was constantly supplied. All the way from one to fifty individuals were placed in a Petri dish during starvation, with the same results in all instances.

When starved in this manner, *Termopsis* begins to lose its large, dominant and principal wood-ingesting protozoön, *Trichonympha* (Fig. 1), by the end of the third day, and by the end of the fourth day perhaps half the individuals of this genus originally present are dead, although few, if any, of the other genera of protozoa have died. By the end of the fifth day, some termites have lost all their *Trichonympha*, while others still retain a few slowly moving, apparently weak individuals. By the end of the sixth day, no *Trichonympha* can be found in any termites, and perhaps half the individuals of the next largest protozoan, *Leidyopsis* (Fig. 2), have died. And in a few termites perhaps all or nearly all of *Leidyopsis* may be dead, but this is exceptional. Also, by this time a few individuals of the next smallest genus, *Trichomonas* (Figs. 5, 6), may have died, but not many. If starvation is continued through the seventh day, some termites completely lose their infection of *Leidyopsis*, while others harbor a few individuals until near the end of the eighth day. After eight days of starvation, then, the two large protozoa, *Trichonympha* and *Leidyopsis*, have all disappeared entirely. If the starvation is continued, *Trichomonas* now begins to die rapidly and by the end of the tenth day nearly all the individuals of this genus are dead, though perhaps one to two per cent. of the total number originally present live sometimes four or five days longer. *Trichomonas* struggles along and dies more slowly than *Trichonympha* and *Leidyopsis*. All of these protozoa feed on the wood particles which their host has eaten, but *Streblo-*

mastix (Fig. 4) does not. It may be dependent on the other protozoa or on the termites for its nourishment. It is difficult to say just when *Streblomastix* begins to die since this genus is much smaller than either of the other three and in the normal or not-starved termite is greatly obscured by the countless thousands of larger individuals, thus making it difficult to determine accurately the normal number present; but the number has perhaps diminished some by the end of the tenth day and by the fifteenth day a great many have died, though not all; some of them, in fact, live almost as long as the termites—three to four weeks.

During starvation most termites are active and appear normal for about fifteen days. However, as soon as many of the protozoa have died, after six days, say, it is not necessary to make a microscopical examination of the intestinal contents to determine what has happened, for there is now much more fluid than formerly present and it looks very muddy—the difference is quite characteristic and cannot be mistaken.

Why do termites, when not given food (wood), lose their protozoa? Do the protozoa die of actual starvation, and most of them much more quickly than their host? Three experiments were carried out which perhaps throw some light on this question. When termites are fed cellulose instead of wood for several months before being starved, and then are cellulose-starved, they lose their protozoa more slowly. For instance, it takes them at least one to two days longer to lose *Trichonympha*. The writer ('25*c*) has shown elsewhere that this termite (*Termopsis*) can live for more than a year and perhaps indefinitely in a perfectly normal manner on a diet of pure cellulose and may it not be true that when it is fed nothing but cellulose (wood is about 50 per cent.) for some time before being starved that it really has more food in its intestine for the protozoa when starvation is begun and for this reason the protozoa are able to live longer? If so, this indicates that when *Termopsis* is wood-starved that its intestinal protozoa, particularly *Trichonympha*, *Leidyopsis*, and *Trichomonas*, die of actual starvation long before their host. We should expect the protozoa to die first since they digest the wood for themselves and their host. And when they die, they perhaps give themselves as food to their host,

which is thus enabled to live considerably longer than its protozoa. In nature, termite protozoa may aid their host by giving themselves as food. It is not known how long the life-cycle of these protozoa is, but, if it is not longer than that of the parasitic protozoa that have been cultivated in artificial media from a single individual, countless millions of them must die daily in a single termite.

b. Oxygenation.

It has been fairly common knowledge for some time that oxygen in rather excessive or abnormal amounts is toxic for many, if not all, forms of animal life. Realizing there must be very little oxygen in the environment of intestinal protozoa, I concluded that they might for this reason be more sensitive to it than their host in an atmosphere of approximately 20 per cent. oxygen. Accordingly, it was decided to determine whether oxygen was more toxic for intestinal parasites than for their host. Obviously, for many reasons, termites are far superior to any other insect and perhaps any other animal for such a study. Workers, soldiers, and nymphs of the reproductive castes, can be counted on to have an infection of 100 per cent., approximately the same in all individuals of the same size and age. And there are millions of very large, active and highly specialized flagellates in a single insect. In fact, nearly half the body weight of the insect is made up of these protozoa. Where, then, could a better opportunity be found to study the effect of oxygen on intestinal flagellates? The termites are easily kept in the laboratory and will live almost indefinitely in tightly corked vials and flasks, for they can stand a very high percentage of CO_2.

These experiments were begun with a different end in view from the use that is made of them in this paper, for it was not thought that all the intestinal protozoa could be removed without injuring the termites too, and still less was the possibility contemplated that oxygen might entirely remove some genera of these flagellates before killing others. The other subject, that of the toxicity of oxygen proper, is being studied now and will be taken up in detail in a later paper.

It was found that if termites (*Termopsis*) were placed in fairly pure oxygen at one atmosphere pressure that all protozoa be-

longing to the genus *Trichomonas* (Figs. 5, 6) were killed within 24 hours and had disappeared from the termites' intestines, while *Trichonympha* and *Leidyopsis*, the two largest genera, and *Streblomastix*, the smallest genus, remained practically unaffected. Of course a few others were probably killed, but not many. The termites were not affected in the least by the oxygen. However, we should not expect them to be for many animals can live in an oxygen atmosphere at this pressure for a much longer period. The surprising thing is that the oxygen kills the protozoa so quickly and removes one genus completely long before the others.

When the termites were confined to the oxygen atmosphere for more than 24 hours, *Trichonympha*, *Leidyopsis* and *Streblomastix* began to die, though not all individuals of these genera were dead until about 72 hours. Sometimes they were dead a little earlier than this and sometimes a few hours later, the variation probably depending on the percentage of oxygen in the particular flask or vial containing them. The oxygen which was used was obtained by heating C. P. $K_2Mn_2O_8$ and was washed in NaOH before being run into the flasks and vials where it displaced most of the air. No effort was made to determine the percentage of oxygen in such an atmosphere, which must have varied considerably at times. Known percentages of oxygen at one and at more than one atmosphere pressure are now being used in the work on toxicity of oxygen for intestinal protozoa. The significant fact is that in all of more than 75 experiments which were carried out the protozoa were all removed in approximately 72 hours. In these 75 experiments, more than 1200 termites were used and none of them, in so far as could be determined by careful observation, ever suffered any ill effects from the oxygen. They easily live eight to ten days in an oxygen atmosphere which kills their intestinal protozoa in three days. No effort was made to determine just how long they would live, for after their intestinal protozoa have been taken from them, they cannot live more than three to four weeks in air.

c. Oxygenation and Starvation.

It was noticed in the starvation experiments that *Trichonympha* disappeared entirely after the termites were starved six days and that *Leidyopsis* had disappeared entirely by the

end of the eighth day of starvation. In the oxygenation experiments, *Trichomonas* disappeared entirely within 24 hours. It was obvious, then, that a combination of these two methods for removing the protozoa would yield interesting results. Accordingly, ten experiments, using approximately 400 termites, were carried out as follows: The termites were oxygenated, as in previous experiments, for 24 hours. This removed all protozoa belonging to the genus *Trichomonas*. About 100 of these termites were starved for six days, and 100 for eight days. In this manner, the first 100, or those individuals which were starved six days, were freed of *Trichonympha*, and the second 100, or those individuals which were starved eight days, were freed of *Trichonympha* and *Leidyopsis*. Thus, in those individuals that were starved eight days, *Streblomastix* only remained, while in those individuals that were starved six days, *Leidyopsis* and *Streblomastix* remained.

d. Wood-feeding after Intervals of Starvation and Oxygenation.

By starvation and by oxygenation and by a combination of starvation and oxygenation we have seen how it is possible to shift the protozoa about almost any way we wish. For instance, we can take out *Trichonympha* by starving six days and leave *Leidyopsis*, *Trichomonas* and *Streblomastix* uninjured; by starving eight days we can remove *Trichonympha* and *Leidyopsis*, leaving *Trichomonas* and *Streblomastix*, and then by oxygenating 24 hours we can remove *Trichomonas*, leaving only *Streblomastix*; or we may oxygenate first and remove *Trichomonas* which will leave *Trichonympha*, *Leidyopsis* and *Streblomastix*, and then if we starve these individuals for six days we have *Leidyopsis* and *Streblomastix* remaining; and by oxygenating for 72 hours all protozoa are removed. By this crisscross procedure we may obtain termites with five[1] protozoal combinations and protozoa-less termites as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*. *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. Thus a wonderful opportunity is afforded for studying the relation of each of the four genera of

[1] Two more combinations have been obtained recently. See addenda for a tabulation of all combinations.

protozoa to its host and to its neighbors or fellow protozoa, for none of these six groups of termites was injured in the least by the methods employed in removing the protozoa. Each group would feed on wood just as it did before the protozoal alterations were made.

Each of these six groups was now fed wood and kept to itself in the same environment of temperature, moisture, and light. Each group contained about fifty individuals. Controls, or termites that had not been treated in any way, were also kept with these five groups. The results of feeding wood to each group may be briefly stated as follows:

(1) *Termites with Leidyopsis, Trichomonas and Streblomastix.*—In normal termites in nature, *Trichonympha* for some reason is perhaps 1000 times as numerous as *Leidyopsis*, and this ratio is fairly constant, although we do not know what makes it so. *Trichonympha* is the dominant genus in size at least and probably in number too—*Trichomonas* and *Streblomastix* may sometimes be as numerous as *Trichonympha*, but they are much smaller. A most interesting thing happens to *Leidyopsis* when its dominant neighbor, *Trichonympha*, is killed; it multiplies rapidly, soon increases very greatly, indeed, in number, and in 20–30 days has filled up the space made vacant in the termite's intestine by the removal of *Trichonympha*. This condition remains permanently and the group of termites is able to live indefinitely on a wood diet. *Leidyopsis*, then, is not only able to take the place of the dominant *Trichonympha* in number but can also take its place as the chief symbiont. As we shall see when we come to study the group of termites with *Trichomonas* and *Streblomastix*, or the group with *Streblomastix*, *Trichonympha*, in nature, is by far the most important symbiont. We know this, even though we were not able to get a pure culture of this genus, because *Leidyopsis* is present in too small a number to be of much importance and *Trichomonas*, as group (3) shows, is not of very great moment as a symbiont. So, in nature, *Trichonympha* is of most value to termites, because for some reason it is dominant over *Leidyopsis*; although, under experimental conditions, *Leidyopsis* can become of as great value to its host as *Trichonympha* is in nature.

(2) *Termites with Trichomonas and Streblomastix.*—When

Leidyopsis, although it is usually never present in nature in nearly such large numbers as *Trichomonas* and *Streblomastix*, is removed the same thing happens to *Trichomonas* that happens to *Leidyopsis* when *Trichonympha* is removed, namely, *Trichomonas* multiplies rapidly and increases very greatly in number for about 30 days, but it never completely fills the intestine of its host. These termites are active for approximately 60–70 days on the average, but after this period many of them die, although some of them live considerably longer and a very small percentage may be able to live indefinitely. However, under the present conditions, we may conclude that the symbiosis between *Termopsis* and its intestinal protozoa is very greatly damaged by the removal of *Trichonympha* and *Leidyopsis*. *Trichomonas*, under these experimental conditions, is undoubtedly of some value to its host, though certainly not as much as either *Trichonympha* or *Leidyopsis*. It is able to keep its host alive for at least 40–50 days, for when it is removed, as we shall see in group (3), the termites die 40–50 days earlier. It is possible that *Trichomonas* in this case has to support *Streblomastix*, as well as its host, for *Streblomastix* cannot live alone as group (3) shows. *Streblomastix* now, as was not the case when only *Trichonympha* had been removed, increases in numbers considerably, and, if a method for removing it without removing *Trichomonas* at the same time were available, *Trichomonas* might then be able to keep practically all of its hosts alive indefinitely. This, however, being a possibility which has not been tested, more work must be done before we can speak with exactness upon the precise ability of *Trichomonas* to keep its host alive.

(3) *Termites with Streblomastix.*—*Streblomastix* does not increase in number; on the contrary, it gradually diminishes, and its host dies within three to four weeks, just as when all protozoa are removed. We conclude, then, that *Streblomastix* is not a symbiont, for it does not seem to be of any value to its host. Incidently, this protozoön, unlike the other three genera, does not ingest wood particles from the intestine of its host, which also suggests that it plays no part in digesting food for its host. *Streblomastix* may depend on the other protozoa for its support.

(4) *Termites with Trichonympha, Leidyopsis, and Streblomastix.*—These termites are able to live indefinitely and it is

not possible to note any change in their protozoa. Perhaps *Trichonympha* takes the place of *Trichomonas*, but if this occurs, it cannot be detected because there are so many *Trichonympha* anyway. The loss of *Trichomonas* is of no consequence.

(5) *Termites with Leidyopsis and Streblomastix.*—*Leidyopsis* multiplies in this group as in group (1), and perhaps takes the place of *Trichomonas* as it does of *Trichonympha*. These termites, as those in group (1), are able to live indefinitely. Since *Streblomastix*, as group (3) shows, is of no value to its host, we may conclude that *Leidyopsis* alone, without the modicum of assistance from *Trichomonas* such as it received in group (1), is able to keep it shost alive indefinitely.

(6) *Termites with No Protozoa.*—These termites eat wood just as those in the other five groups, but are not able to live longer than three to four weeks. The inability to maintain themselves on their normal diet of wood is caused by the removal of the intestinal flagellates, particularly *Trichonympha* and *Leidyopsis*, from them.

Discussion.

Intestinal protozoa must live in an environment with a smaller percentage of oxygen than their hosts, and should, therefore, experience the greater difficulty when the oxygen environment of the host is raised from 20 to 100 per cent., provided, of course, the oxygen percentage of the parasite's environment does not increase correspondingly with that of its host. For instance, if there is normally, say, 1 per cent. of oxygen in the parasite's environment and 20 per cent. in that of the host, when the host is placed in an atmosphere of 100 per cent., the percentage of the host's environment is thus increased only five times, while that of the parasite is increased eighty times. On the other hand, if the oxygen percentage of the parasite's environment increases correspondingly to that of its host, or, as in this case, five times, then the parasite would be in 5 per cent. oxygen when its host was in 100. When termites are placed in 100 per cent. oxygen, the oxygen percentage of their parasite's environment may be increased much more than their own, and the parasites are killed, just as any animal would be with so great an oxygen increase. If this is true, the parasites can undergo as great change in

oxygen as their host, and oxygen is really not any more toxic for them than for their host. They die, then, while their host is uninjured, because the oxygen percentage of their environment increases many times more than that of their host.

In larger animals with a different system of respiration, the oxygen percentage of host and parasite may increase correspondingly, in which case it would be impossible to kill the parasites of, say, a vertebrate by confining it in an oxygen atmosphere without at the same time killing the vertebrate, unless, of course, oxygen is actually more toxic for the parasites. At any rate, other parasites may be killed by the use of oxygen if we can develop a method of getting it to them.

When termites are starved, their largest protozoa die first, but when they are oxygenated, their next to the largest one dies first. What is the cause of this? The larger and more active ones may require more food than the smaller ones, and for this reason starve more quickly, if starvation is the actual cause of death. Or it may be that the smaller ones are partly nourished by their host or by their larger protozoan neighbors. In the case of oxygenation, the smaller ones may die first because of the higher ratio of surface-volume exposure which they have.

One interesting problem which this study brings out is, what maintains the fairly definite ratio between the four genera of protozoa? The host may produce a reaction product for each genus which inhibits its multiplication beyond a certain point. However, this does not seem very likely, for when *Trichonympha* is removed, *Leidyopsis* takes its place, and when *Leidyopsis* is removed, *Trichomonas* partly takes its place. The protozoa may inhibit the reproduction of each other beyond a certain point. And another possibility is the question of struggle for food which must go on where such a large number of protozoa are present.

Summary and Conclusions.

The termite which was used in these experiments belongs to the genus *Termopsis*. Very probably two species, *T. nevadensis* Hagen and *T. angusticollis* Hagen, have been used. These two species are so nearly alike that they can be distinguished at present only by a study of their winged adults, which were not

present in most of the material used. The two species probably harbor an identical protozoan fauna.

Four genera of protozoa are invariably present in these termites. These in order of size are: *Trichonympha* (Fig. 1), *Leidyopsis* (Fig. 2), *Trichomonas* (Figs. 5, 6), and *Streblomastix* (Fig. 4). Kofoid and Swezy ('19) claim that another genus, *Trichomitus* (Fig. 3), is also present, but, for reasons given in this paper, it is impossible to say whether or not *Trichomitus* is present. If it is, it reacted in every way as *Trichomonas* did, and has the same relation to its host and fellow protozoa as *Trichomonas*.

Two methods, starvation and oxygenation, for removing the protozoa are given. By using each method separately and by a combination of the two it was possible to get five different combinations of the protozoa, without injuring the termites in the least. By starving for 6 days, *Trichonympha* was removed entirely; by starving for 8 days, *Leidyopsis* was removed completely; by oxygenating for 25 hours, *Trichomonas* was entirely removed; by oxygenating for 24 hours and starving for 6 days, *Trichomonas* and *Trichonympha* were removed; by oxygenating for 24 hours and starving for 8 days, *Trichomonas*, *Trichonympha* and *Leidyopsis* were removed; by oxygenating for 72 hours, all protozoa were removed. From this we get one group of termites with no protozoa in them and five groups with a different combination of protozoa in each group as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*, *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. By feeding the normal diet of wood to each of these groups of termites it was possible to work out the relation of each protozoan genus to its host and to its neighbors or fellow protozoa.

When group (1), which contained *Leidyopsis*, *Trichomonas* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied rapidly, increased greatly in number and was able in 20–30 days to fill the vacant space made in the host's intestine when *Trichonympha* was removed. This group is able to live indefinitely.

When group (2), which contained *Trichomonas* and *Streblomastix*, was fed wood, *Trichomonas*, like *Leidyopsis* in group (1), multiplied rapidly and increased greatly in number for about 30

days, but was never able to fill the intestine with protozoa, that is to say, *Trichomonas* was never able to entirely take the place of *Trichonympha* and *Leidyopsis* in volume. Most of the termites of this group were able to live 70–80 days and some of them longer, although very few, if any, were able to live indefinitely. *Trichomonas*, then, is of some value to its host as a symbiont. It can keep its host alive 40–50 days longer than the host would be able to live without it. If *Streblomastix*, which is certainly not of any value to its host and may have to be supported by *Trichomonas* in this group, were not present, *Trichomonas* might be of more value to its host.

When group (3), which contained *Streblomastix*, was fed wood, death resulted within three to four weeks, the same time it occurs when all protozoa are removed. *Streblomastix* did not multiply at all; on the contrary, it gradually diminished in number. This protozoön, then, is not a symbiont. It may either receive its nourishment from its host or from the other protozoa directly, probably the latter.

When group (4), which contained *Trichonympha*, *Leidyopsis* and *Streblomastix*, was fed wood, all individuals were able to live indefinitely. The removal of *Trichomonas* did not seem to affect the symbiosis at all. *Trichonympha* perhaps took the place of *Trichomonas* very quickly.

When group (5), which contained *Leidyopsis* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied and increased in numbers just as it did in group (1), perhaps taking the place of *Trichomonas*—though this could not actually be seen—just as it did that of *Trichonympha*. These termites were able to live indefinitely. *Leidyopsis*, even though in nature its ratio to *Trichonympha* is approximately 1 : 1000, can take the place of *Trichonympha* under experimental conditions both in number and in ability to keep its host alive indefinitely. In nature, however, *Trichonympha* must be the chief symbiont, for it is so much more numerous than its closest neighbor, *Leidyopsis*.

When group (6), which contained no protozoa, was fed wood, death resulted within three to four weeks.

The results of these experiments, as regards the symbiotic relationship between termites and their intestinal protozoa, are in accord with those obtained by the incubation method (Cleve-

land, '24*a*). The symbiosis between these insects and their intestinal flagellates has now been clearly demonstrated by three entirely different methods, namely, incubation, starvation, and oxygenation.

ADDENDA.

Since this paper went to press sometime ago several additional observations of interest have been made.

Thirteen colonies of *Termopsis nevadensis* and six colonies of *Termopsis angusticollis* have been obtained. The protozoa in all of these are the same.

By dry fixation and staining with Wright's stain one and three minutes respectively it has been possible to demonstrate beyond question four anterior flagella on *Trichomonas*. No organisms with three anterior flagella, the number *Trichomitus* is said to possess, have been observed. Hence it is quite probable that *Trichomitus* does not occur in *Termopsis*. Another interesting result was obtained by this method of fixation and staining: It was discovered that *Streblomastix* has only four flagella, instead of six, the number figured by Kofoid and Swezy ('19). The flagella stand out as big red lines which may be counted almost as easily as so many red pencils. Perhaps this method of fixing and staining will prove valuable in determining the flagella number of other protozoa.

By oxygenating *Termopsis* at a pressure of 1.5 atmospheres for 7 hours it has been possible to remove both *Trichomonas* and *Streblomastix* from many, though never all, hosts without seriously effecting *Trichonympha* and *Leidyopsis*. When termites oxygenated in this way are starved for six days, *Trichonympha* disappears entirely, thus making it possible to obtain termites with only *Leidyopsis*. This gives two more protozoal combinations, in addition to the five already obtained, *viz.* termites with *Trichonympha* and *Leidyopsis*, and termites with *Leidyopsis*. It now seems desirable to tabulate all the protozoal combinations; show how they were obtained, and how, if at all, they effect their hosts.

Table I.

Results of Various Methods which Have Been Employed in Removing One or More Genera of Protozoa from the Large Pacific Coast Termite, *Termopsis nevadensis* Hagen.

Every host in nature always harbors each genus. — = absent, *i.e.*, treatment killed all protozoa of this genus and + = present, *i.e.*, treatment had no effect.

Methods of Treatment.	The Protozoa.				Result of Treatment on Host.
	Trichonympha.	*Leidyopsis.*	*Trichomonas.*	*Streblomastix.*	
1. Starvation for 6 days	—	+	+	+	Lives indefinitely.
2. Starvation for 8 days.	—	—	+	+	Lives about 10 weeks.
3. Oxygenation for 24 hours at 1 atm. . . .	+	+	—	+	Lives indefinitely.
4. Oxygenation for 24 hrs. at 1 atm. Starvation for 6 days.	—	+	—	+	Lives indefinitely.
5. Oxygenation for 24 hrs. at 1 atm. Starvation for 8 days.	—	—	—	+	Lives 3–4 weeks.
6. Oxygenation for 7 hrs. at 1.5 atms. . .	+	+	—	—	Lives indefinitely.
7. Oxygenation for 7 hrs. at 1.5 atms. Starvation for 6 days	—	+	—	—	Lives indefinitely.
8–13. Oxygenation, 1 atm., 72 hrs., 1.5, 9 hrs., 2, 5 hrs., 2.5, 2 hrs., 3, 1 hr and 5 min., 3.5 40 min.	—	—	—	—	Lives 3–4 weeks.

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Explanation of Plate I.

The Protozoa of *Termopsis*. Figs. 1–4 after Kofoid and Swezy; Figs. 5, 6 original.

Fig. 1. *Trichonympha campanula*. × 300.

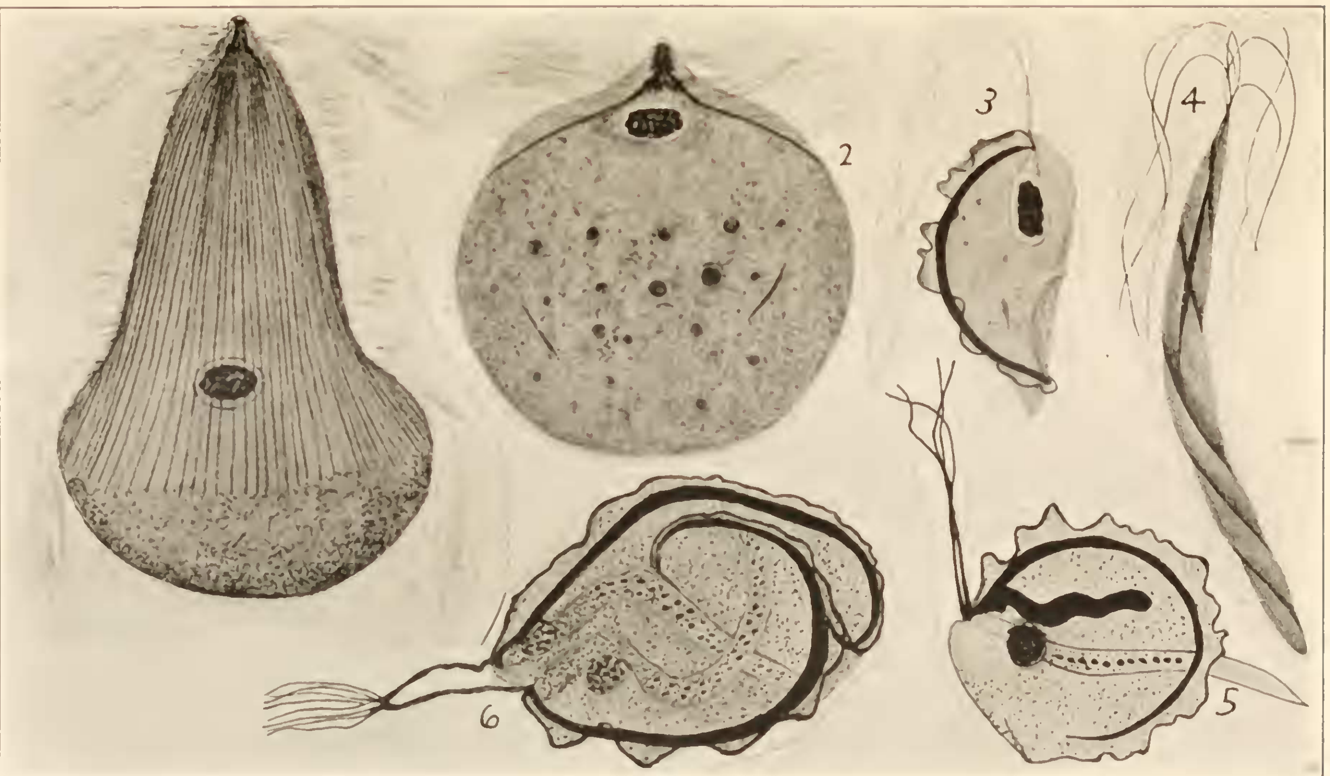
Fig. 2. *Leidyopsis sphaerica*. × 300.

Fig. 3. *Trichomitus termitidis* which may be a synonymn of *Trichomonas termopsidis* (Figs. 5, 6). × 625.

Fig. 4. *Streblomastix strix*. × 2500.

Fig. 5. *Trichomonas termopsidis* fixed in weak Flemming's fluid. Note parabasal body which is not present in Fig. 6 because this organism was fixed in Schaudinn's fluid which nearly always makes the parabasal invisible. × 1440.

Fig. 6. *Trichomonas termopsidis* dividing form fixed in Schaudinn's fluid. Paradesmose has just disappeared. Note doubling of nuclei, axostyles, anterior flagella, undulating membrane, and chromatic basal rod of undulating membrane. Parabasal body does not appear because of fixation in Schaudinn's fluid. × 1440.



L. R. CLEVELAND.

SOME PHYSIOLOGICAL DISTINCTIONS BETWEEN FRESHWATER AND MARINE ORGANISMS.

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I.

Aquatic organisms are in general distinctly divided into those species inhabiting fresh water and those inhabiting sea water. Yet this division is evidently not a phylogenetic one, since closely related species live in either (Quinton, '04). Only in a few species are the same individuals capable of passing from one medium to the other. The transition zones of brackish water are relatively small in extent and very variable, still there are a few organisms which thrive only in such diluted sea water.

Particularly the distribution of fishes between fresh water and sea water has excited the interest of biologists. Paul Bert tried to find why fish died when taken from their usual medium and placed in the other ('71, '73). For marine animals he ('83) reached one important conclusion, namely, that the toxic effect was not always due to the dilution of the substances dissolved in sea water. This conclusion has been thoroughly confirmed by the subsequent observations of Loeb ('03) and others.

Practically no further analysis of this problem could be made without careful quantitative studies. Such were undertaken by Sumner ('06) upon marine teleost fishes. He found significant changes in the water content and in the salt content of the animals during their immersion in diluted sea water. Thus the deleterious effect seemed to be a loss of essential body constituents, because the animals were unable to regulate favorably their integumental permeability under the new circumstances.

Meanwhile a new line of attack came to light with the discovery of salt antagonisms by Ringer ('82). Loeb ('00) found that marine organisms were readily affected by the disturbances of the balance between salts, but were almost independent of osmotic pressure changes. Thus ('03), the marine crustacean *Gammarus* endured dilution of the seawater medium just as well

if the diluting fluid was distilled water as if it was a sugar solution isotonic with sea water.

It is important to realize that various groups of animals differ greatly in their ability to control the interchange of substances between body fluids and external environment (Semper, '80). In all metazoa the covering epithelial layer of the body carries on an active regulation of the interchanges, so that the outer medium affects the more vital tissues only after it has with surprising slowness changed the composition of the internal medium (Adolph and Adolph, '25). Thus in all these animals there are two lines of defense, two regulating surfaces which must be penetrated. To illustrate several grades of this ability to control the interchanges at the body surface, we may cite the mammals, in which it is well known that the skin is impermeable at all times; the aquatic amphibia, in which the permeability is controlled in such a way that significant chemical interchanges between body and environment are strongly opposed; and the annelid worms, in which the interchanges are still less selected. In the teleost fishes essential interchanges are limited to the gill surfaces (Sumner, '06). In elasmobranch fishes the entire skin is partially permeable, while in invertebrates the body fluid often interchanges freely with the medium.

We have attempted to compare the vital resistance to the penetration of chemical agents in freshwater animals and marine animals. For this purpose we have chosen those animals whose entire body surface is normally permeable in some degree, namely, small invertebrates. In them the surface mass ratio is sufficiently large that effects of the chemical environment quickly manifest themselves.

II.

An excellent measure of the amount of integumentary regulation performed by organisms is the rate of change of body weight when the organisms are transferred from their usual medium to a solution of different chemical composition (Adolph and Adolph, '25). Thus, when freshwater animals are immersed in salt solutions there is a rather sudden loss of water, so that the body volume quickly reaches a new lower level. Seawater animals placed in diluted sea water gain in volume, even though the

dilution be carried out with an isotonic solution of a non-electrolyte. Several species of invertebrates, both freshwater and marine, have been compared in this manner. The flatworm *Dendrocœlum* and the earthworm *Lumbricus*, both of which inhabit fresh water, reach the new volume level in about 5 hours. The marine annelid *Phascolosoma*, when placed in sea water diluted to half its normal concentration, attained the new volume level in less than 2 hours. Evidently *Phascolosoma* adjusts to the new environment more rapidly than the freshwater species; it has less resistance to the penetration of water through its surface.

We wished to know whether dissolved substances likewise penetrated at different rates through the body coverings of these two classes of animals. After the first adjustment to the immersion in salt solution by loss of water, freshwater animals remained at the new level of volume for several days, and when replaced in fresh water recovered two thirds of the original loss. *Phascolosoma*, however, in diluted sea water lost slowly a considerable amount of the dissolved substance of the body fluids, so that when finally returned to normal sea water very little water passed from the body compared to that originally gained.

A still larger number of animals we have compared with respect to their resistance to solutions of salts and other substances. A list of these species, and the highest concentrations of certain solutes which they can just survive for a considerable length of time, are given in Table I. An important conclusion can immediately be drawn from these data; namely, that for freshwater organisms the osmotic pressure of the medium usually limits survival, while for marine organisms a great range of concentrations can be resisted. Thus, marine Gammarus will live indefinitely if transferred to sea water diluted with distilled water up to 0.5 per cent. (0.005 M), or concentrated by the addition of salts up to 160 per cent. (1.56 M, corrected for ionization). Freshwater *Gammarus*, on the other hand, are usually killed by immersion in any solution of a concentration equivalent to 0.35 M. Gradual dilution or concentration of the medium does not appreciably extend these limits.

The general significance of the conclusions from all the above experiments we interpret to be that both dissolved substances

TABLE I.

MAXIMUM SURVIVAL CONCENTRATION OR PERCENTAGE OF VARIOUS DISSOLVED SUBSTANCES WHICH WERE ENDURED BY 3 FRESHWATER SPECIES AND 3 MARINE SPECIES AT 22° C. FOR THE ARBITRARY TIMES SPECIFIED.

Figures in parenthesis are molar concentrations, corrected for ionization.

Solutions in Distilled Water.	Sea Water.	Urea, M.	Glycerol, M.	Glucose, M.	Sucrose, M.	NaCl, M.	KCl, M.	$CaCl_2$, M.	Ringer,[1] M.	$MgCl_2$, M.
Gammarus fasciatus (Crustacean) 5 hrs.....	100% (0.97)	0.35	0.35	0.38	0.30	0.20 (0.35)	0.04 (0.07)	0.03 (0.08)	0.26 (0.43)	0.14 (0.35)
Phagocata gracilis (Flatworm) 24 hrs.....	35% (0.33)	0.32	0.32	0.32	0.23	0.16 (0.28)	0.04 (0.07)	0.04 (0.11)	0.15 (0.26)	0.10 (0.25)
Paramecium caudatum (Protozoan) 1 hr.......	17% (0.16)	0.32	0.25	0.20	0.20	0.11 (0.20)	0.07 (0.13)	0.05 (0.14)	0.11 (0.20)	0.12 (0.30)
Isotonic Solutions Added to Sea Water.	Distilled Water, %.	0.88 M Urea, %.	0.88 M Glycerol, %.	0.88 M Glucose, %.	0.88 M Sucrose, %.	0.54 M NaCl, %.	0.54 M KCl, %.	0.30 M $CaCl_2$, %.		
Gammarus locusta (Crustacean) 5 hrs.....	99½	70	99½	65	85	80	5	21		
Procerodes wheatlandi (Flatworm) 24 hrs.....	98	70	97	90	95	50	4	40		
Copepod (Crustacean) 1 hr......	60	60	60	50	60	60	3	15		

[1] Unbuffered Ringer's solution was made by mixing 97 volumes of 0.54 M NaCl, 2 volumes of 0.54 M KCl, and 1 volume of 0.30 M $CaCl_2$. This was taken to be 0.54 M.

and water penetrate the tissues of marine animals faster than they pass through the covering layers of freshwater animals. But in both cases the presence of salts in abnormal proportions in the medium renders the permeability of the integument high.

The greater permeability to salts and water in marine organisms accords with the ordinary conditions of existence for these animals. Marine animals are constantly bathed in a medium which is physiologically as suitable in inorganic composition as any internal one (Fredericq, '22). Freshwater animals, on the other hand, cannot afford to lose dissolved substances from their bodies nor to allow the entrance of water into their bodies up to the point where osmotic equilibrium would result.

Osmotic pressure changes *per se* are evidently not particularly deleterious to the vitality of internal tissues, providing the integument does not attempt to regulate against them. In marine organisms the freer penetration of solutes allows the internal medium to keep pace with the outer medium as regards composition, and so long as the salt balance is preserved, no essential functions are inhibited.

Morphologists have often attributed the chemical resistance of freshwater organisms to their possession of an outer cuticulum. Perhaps the resistance referred to is the resistance to the penetration of protein-precipitating agents, and in this a proteinaceous cuticulum probably assists. But under ordinary conditions the cuticulum is by no means either impermeable or semipermeable. Rather the living integument is responsible for the maintenance of restricted or selective permeability.

III.

A second significant characteristic of freshwater animals appears to be that their body fluids have a lower osmotic pressure than those of marine organisms.

Whether there is a relation between toxicity and tonicity we have attempted to investigate by measuring the survival of plasmolyzed *Spirogyra* filaments. With a variety of plasmolysing agents it was found that the toxic concentration was almost exactly the lowest one which produced distinct permanent plasmolysis. By gradually increasing the concentration of the medium, both toxicity and plasmolysis were prevented. Now, in

all the freshwater animals studied, toxic effects followed immersion in a concentration of about 0.35 M non-electrolyte solutions, or the equivalent concentration of electrolytes. Fredericq ('98) and Botazzi ('08) have shown that the tissue fluids of freshwater animals always have osmotic pressures less than half of that of sea water ($\Delta = 0.8°$ C.), and usually have only one eighth to one fourth of that of sea water; while marine animals have body fluids which are exactly isotonic with sea water. We can thus probably regard the maximum survival concentration for freshwater animals as a measure of the osmotic pressure of their body fluids. All the freshwater animals studied evidently had, therefore, internal osmotic pressures equivalent to 0.20 to 0.35 M.

The acclimatization of marine organisms to changed osmotic conditions contrasts to that for freshwater organisms. In several instances marine animals placed in glass-distilled water died, yet most of them survived a mixture of 98 or 99 per cent. of distilled water with only 2 or 1 per cent. of sea water. In other words, most marine animals are able to live after abrupt change to almost pure fresh water, providing that the remaining salts are present in physiological proportions. Gradual dilution of the sea water over several days did not materially help marine animals to endure pure water, though complete acclimatization has been secured over long periods of time by other observers (Beudant, '16, Plateau, '71, Semper, '80).

For freshwater organisms, gradual increase in the concentration of the medium did not greatly increase the maximum survival concentration. It is evident from this that plasmolytic effects are not the important ones in producing this toxicity. In diluted sea water all the freshwater animals studied except *Gammarus* were killed in concentrations less than half of that of the sea-water at Woods Hole ($\Delta = 1.81°$ C.), and acclimatization never increased this toxic limit up to half of the concentration of the sea water. Similar acclimatizations to specifically poisonous substances have been demonstrated by Davenport and Neal ('96) and numerous other investigators, so that it seems doubly certain that the ultimate toxic effect is not plasmolytic. Moreover, the toxic effect cannot be attributed to sudden volume or concentration changes such as are brought about by diffusion. Certain balanced solutions such as sea water can be resisted in

spite of their high concentration by freshwater *Gammarus*; there is no interference with the regulatory activity of the integument. At this point we approach the problem of the conditions for the survival of the internal tissues, which are evidently very different from the conditions which the external medium may impose when an integument is interposed.

IV.

It appears, therefore, that freshwater organisms are strongly contrasted to marine organisms with respect to their ability to adjust to changes in their chemical environment. Marine organisms in general maintain a higher degree of interchange of inorganic materials with their surroundings. Freshwater organisms, on the other hand, have a more restricted and selective interchange, and thus dissolved materials are kept inside their bodies and water is kept outside. In protecting themselves from their environment through the retention of salts, freshwater organisms have laid themselves open, paradoxically, to osmotic disturbances of the integument. Marine organisms, by allowing the environing medium to serve as the physiological fluid, are able to endure a much greater change in the environing medium, than are freshwater organisms with their greater regulation of the internal medium. This greater change is one of concentration only, however, for both groups of animals are equally susceptible to variations in the proportions of salts in the medium. This condition in the freshwater animals is entirely due to the peculiar and variable type of permeability found in the integuments of this group, whereby they normally maintain a concentration difference between outside and inside fluids, as has been pointed out previously (Quinton, '04, Adolph and Adolph, '25). This activity of the integument serves excellently in the normal medium, but adds a factor of susceptibility.

Existence in the freshwater medium is accompanied by the possession by all freshwater organisms of relatively dilute tissue fluids. Apparently it is the integumentary activity which is upset by increased concentrations of the medium. Its upset, in turn, produces rapid changes of a deleterious nature in the composition of the body fluids. Marine organisms, on the other hand, have body fluids which are in complete chemical equilib-

rium with their environments (Quinton, '04). Thus it turns out that while the essential physiological constitution of the tissues of the two groups of animals does not differ, the limiting factors for the proper functioning of their regulatory integuments differ vitally.

It seems probable that the transition from one type of integumental permeability to the other type occurs automatically during the long process of acclimatization which accompanies the transfer of an organism from one aquatic medium to the other; that both types are inherent in all integuments.

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ENDOCRINE GLANDS AND BILATERAL SYMMETRY: OBSERVATIONS UPON FORELIMB ERUPTION IN FROG LARVÆ UNDER TREATMENT WITH THYROID AND THYMUS EXTRACTS.

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Introduction.

A conspicuous feature of thyroid-accelerated metamorphosis in frog larvæ is the eruption of the forelimbs. Before these become visible externally they are located beneath the skin in the gill chamber. For some years the writer has been aware of the fact that the left forelimb is protruded before the right forelimb, sometimes by as much as several days. This condition was recorded in a previous paper (Jordan and Speidel, '23) and has probably also been noted by other workers in the field of amphibian metamorphosis. In half-grown bullfrog and green frog tadpoles no exceptions to this were seen; *i.e.*, no case appeared of precedent right forelimb eruption in thyroid-treated tadpoles. It was, therefore, of some interest to find in a jar of seven thymus-treated tadpoles one animal in which the right forelimb appeared two weeks before the left. Furthermore, in two other animals in this lot the amount of skin degeneration in the right forelimb region was definitely farther advanced than that on the left side, a condition indicating probable prior right forelimb eruption. Death ensued, however, before the appearance of either forelimb. Two of the other four animals in this jar put out the left forelimb first, and in one the amount of skin degeneration on the left side plainly foreshadowed the prior eruption of the limb of that side. The remaining animal put out both limbs over night. In this jar of seven thymus-treated animals, therefore, the ratio of "right-handed" to "left-handed" animals is 1 : 1.[1]

The question suggests itself as to whether the endocrine secre-

[1] The terms "right-handed" and "left-handed" are used to denote merely prior right forelimb eruption or prior left forelimb eruption, respectively.

tions represented by the thyroid and thymus extracts actually affect in a differential manner the bilateral symmetry of the developing animals. Further experiments were set under way in an attempt to analyze the factors controlling variation in forelimb eruption.

There is a difference of opinion as to the condition in normal frog larvæ. Barfurth ('87) in Europe finds in the case of *Rana fusca* that 80 per cent. put out the right forelimb first, the left almost always following in from two to eight hours. On the other hand, Gudernatsch ('14), using *Rana temporaria* and *Rana esculenta*, states that he has always found the reverse to be the case; *i.e.*, in about 80 per cent. the left forelimb erupts first.[2] Dickerson ('20) observes that in normal bullfrog and green frog metamorphosis the left forelimb is usually put out first. My own observations upon the normal tadpoles of *Rana sylvatica*, *Rana clamata*, *Rana catesbeiana*, and *Hyla crucifer* lead me to agree with Gudernatsch and Dickerson that in a definite majority of cases the left limb is the first to erupt.

Material, Experiments and Observations.

The material used includes about 800 tadpoles of *Hyla crucifer*, *Rana catesbeiana*, *Rana clamata*, and *Rana sylvatica*, and a few of *Rana cantabrigiensis* and *Rana pipiens*. The bullfrog and green frog tadpoles were collected at Charlottesville, Virginia, the others at Woods Hole, Massachusetts. Untreated animals were usually kept in aquaria containing pond water and weed. Administration of endocrine extract was accomplished by placing some of the extract in the water with the animals. Thyroid and thymus desiccated extracts were used.

The accompanying table indicates the relative frequency of left or right forelimb eruption, as it occurs under normal conditions, under thyroid treatment, and under thymus treatment.

[2] Gudernatsch makes this statement in a footnote referring to his experiment with thyroid-accelerated metamorphosis. It is probable, therefore, that he included his observations on thyroid-treated animals with those on normal animals in regard to forelimb eruption, not realizing that thyroid treatment affects forelimb eruption. Thyroid administration, as shown by this paper, markedly favors the prior eruption of the left forelimb. His percentage, therefore, is not correct, but is too high in favor of lefthandedness.

TABLE I.

In this table is given for each species of tadpole the number of individuals observed with prior left forelimb eruption as compared with the number of individuals observed with prior right forelimb eruption, under normal conditions, under thyroid treatment and under thymus treatment.[3]

	Normal-untreated.		Thyroid-treated.		Thymus-treated.	
	Prior Left.	Prior Right.	Prior Left.	Prior Right.	Prior Left.	Prior Right.
Hyla crucifer	43 (72%)	17 (28%)	25 (100%)	0	17 (50%)	17 (50%)
Rana sylvatica	42 (65%)	22 (35%)	19 (100%)	0		
Rana catesbeiana and *R. clamata*	3	0	85 (100%)	0	3 (50%)	3 (50%)
Rana cantabrigiensis			4 (100%)	0		
Rana pipiens			20 immature tadpoles, all died before either forelimb erupted			
Total, all species	88 (70%)	39 (30%)	133 (100%)	0	20 (50%)	20 (50%)

Under normal conditions a definite majority of tadpoles puts out the left forelimb first; in *Hyla* 72 per cent., in *Rana sylvatica* 65 per cent. The few observations upon untreated bullfrog and green frog tadpoles in the three-limb condition support Dickerson's observation that the left limb usually erupts first. Under thyroid treatment the left forelimb is invariably protruded first in all species studied, if those putting out forelimbs during the first forty hours of the treatment are excluded. Under thymus treatment apparently a 1 : 1 ratio is indicated. The experiments with *Hyla* afford the best comparative figures and may be taken as typical.

With thyroid treatment of half-grown tadpoles 100 per cent. put out the left forelimb first. A special experiment was tried in which 75 *Rana sylvatica* tadpoles were subjected to thyroid treatment, many of these at the time being on the verge of putting out the forelimbs. Among those animals putting out forelimbs within the first forty hours, seven righthanded ones were seen; thereafter all were lefthanded. It may be concluded that in animals protruding the forelimbs within this time, an original bias toward righthandedness may not be changed. In a similar experiment with 25 *Hyla* tadpoles that were also fairly close to

[3] For reasons explained in the text all animals putting out forelimbs within the first two days of thyroid or thymus treatment are discarded, and do not figure in the table.

the time of forelimb eruption, one righthanded animal occurred during the first 24 hours: after that all were lefthanded. For this reason, in Table I. all animals putting out forelimbs during the first two days are omitted from the reckoning, since the original normal bias may not have been sufficiently influenced by the endocrine extract.

How long it takes for the thymus extract to affect an original bias is not known. Since it is probably not so powerful as the thyroid it may be that more than the first two days' results should be discarded. If the first two days are discarded the ratio is 17 : 17 as given in the table. If the results of the first three to seven days are discarded the ratio shifts progressively to favor righthandedness. Only a much larger number of animals under observation would give a trustworthy ratio. The figures given, however, are in all probability enough to show that the normal ratio has been affected and shifted in the direction of righthandedness.

Interpretation and Discussion.

These observations leave no doubt that the bilateral symmetry of the developing frog tadpole, as indicated by the relative time of forelimb eruption, is influenced by thyroid extract; possibly also by thymus extract. The action of the thyroid extract will first be discussed. The blood carries the active thyroid principle to all parts of the body. It is inconceivable that the thyroid autacoid should have one effect on the tissues on the left side and another effect on exactly similar tissues on the right side. Therefore, the effect of the thyroid in changing the normal symmetrical development must be due to some original fundamental asymmetry of the body pattern.

The following findings are pertinent: The tadpole is conspicuously asymmetrical in respect to its respiratory apparatus. A spiracle, or outlet from the gill chamber, is present on the left side only (Fig. 1). This outlet drains both left and right gill chambers, these being connected by a canal across the mid-line. The forelimb is present in the gill chamber, its degree of development depending upon the general developmental state of the tadpole. Both in normal and thyroid-induced metamorphosis

the left limb is pushed through the spiracle, a variable amount of preliminary skin degeneration occurring. The right forelimb erupts through the skin only after the latter has undergone a certain amount of degeneration. This degeneration starts in the

FIG. 1. FIG. 2.

FIG. 1. Ventral view of a tadpole of the tree frog (*Hyla crucifer*) showing well developed forelimbs (*f*) still imprisoned beneath the skin in the gill chambers (*r.g.c.* and *l.g.c.*). The position of the sinistral spiracle (*s*) is shown, which drains both gill chambers; also the canal (*c*) across the mid-line which connects right and left gill chambers. The dotted line below the forelimbs indicates the position of the partition separating the gill chambers from the abdominal cavity.

FIG. 2. Dorsal view of a thyroid-treated tadpole (*Hyla crucifer*) in typical three-limb stage after the eruption of the left forelimb. Collection and retention of air (*a*) in the right gill chamber causes bulging out of the skin and interferes with forelimb eruption on that side. Excluding the first two days, prior left forelimb eruption occurs invariably.

vicinity of the elbow, and the elbow is usually protruded first. Movements of the forelimb finally enable it to break completely through. In young thyroid-treated tadpoles, however, the forelimbs are little developed so that the elbow is not prominent at the surface. In these cases the hand or whole arm appears as a tiny white stump after the skin degeneration has proceeded far enough.[4]

The administration of thyroid extract to a half-grown tadpole obviously upsets the respiratory mechanism. More air is taken

[4] While this paper was in press, Helff ('24), at the Washington meeting of the American Society of Zoölogists, reported a series of experiments which show clearly that the opercular skin autolysis preceding forelimb eruption is brought about by substances given off by the adjacent atrophying gills.

in. But apparently the animal is not yet very well fitted for utilizing it properly. Small bubbles of air collect in the gill chambers and are not expelled, or expelled with difficulty. This is especially true of the right gill chamber which has no outlet except through the left spiracle (and, of course, the mouth). This proves to be the deciding factor. The left gill chamber is usually drained well enough except in very immature tadpoles, so that skin degeneration and forelimb eruption on that side are not interfered with. On the right side, however, the retention of air in the chamber causes bulging out of the skin (Fig. 2), and interferes to a greater or less degree with the normal eruption on that side, thus bringing about the typical prior left forelimb eruption. A tadpole in this stage does not present a normal posture when at rest, but floats with the right side somewhat elevated owing to the air in the right gill chamber. The difference between a thyroid-treated animal in this condition and either normal or thymus-treated animals may be seen by comparing Fig. 2 with Figs. 3 and 4. Occasionally, in very young

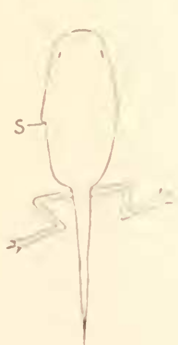

Fig. 3.

Fig. 4.

Fig. 3. Dorsal view of a normal tadpole (*Hyla crucifer*) before the eruption of either forelimb. The location of the spiracle (*s*) on the left side only, leads to a majority (70 per cent.) of prior left forelimb eruptions.

Fig. 4. Dorsal view of a thymus-treated tadpole (*Hyla crucifer*) showing prior right forelimb eruption. Either right or left forelimb may erupt first. In the region of the spiracle (*s*) may be seen an angular projection (*p*) caused by the pressure of the imprisoned left forelimb.

and immature tadpoles no forelimbs erupt with thyroid treatment, the animals dying in the two-limb stage. In these, bubbles of air may be seen in both gill chambers. The pulmonary

development of the animal is not adequate to the demands imposed upon it by thyroid treatment and death results. Coupled with this respiratory disturbance is also the condition of anemia already pointed out elsewhere (Jordan and Speidel, '23). The older the tadpole the more probable it is that both forelimbs will erupt. The pulmonary apparatus is presumably better developed, and the forelimbs are large enough so that limb movements aid both in expelling the air from the gill chamber and in breaking through the skin. In thyroid-treated animals that are near the time for metamorphosis both forelimbs are put out with little trouble, the sinistral location of the spiracle becoming of less importance.

In a recent paper by Swingle ('23) one figure is given to show the effect of iodo-tryosine administration in accelerating metamorphosis in pituitaryless *Rana sylvatica* tadpoles. After sixteen days of treatment the specimen illustrated has one forelimb, that one being a right forelimb. Swingle does not state when this particular right limb appeared but does say that two right forelimbs broke through as early as the eighth day, the average, however, being about twenty days. This is an interesting observation in comparison with my results after thyroid treatment; *i.e.*, 100 per cent. prior left forelimb eruption after the first two days. It seems to mean, either that iodo-tryosine does not affect the respiratory apparatus in the same way as does thyroid extract, or that the pituitary gland plays a rôle also in influencing symmetrical development.

It now remains to discuss the normal condition and the thymus-treated condition. Three factors are considered of chief importance in determining forelimb eruption: (1) sinistral location of the spiracle; (2) relative degree of skin degeneration over the forelimb region on the two sides of the body; (3) relative size and strength of the forelimb. In partly grown thyroid-treated animals the first factor is by far the most important, as has been shown. In mature untreated tadpoles, however, this factor does not remain the all-important one. The forelimbs are now so large that the left one cannot be pushed through the spiracle without a fair amount of previous skin degeneration. Simultaneous skin degeneration occurs on both sides. The size, strength and activity of the imprisoned forelimb now becomes of

much importance. In about 30 per cent. of cases the right forelimb succeeds in overcoming the handicap of having no spiracle to come through, and breaks through by main strength before the left. In the other 70 per cent. the left limb aided by the sinistral spiracle comes through first. A majority in favor of lefthandedness is about what should be expected.

Mention should be made again of Barfurth's results. In normal *Rana fusca* tadpoles he finds that 80 per cent. put out the right forelimb first. Presumably these are like all other frog tadpoles in having sinistral spiracles although Barfurth does not refer to this feature. He believes the prevailing righthandedness of this species is accounted for by two factors: (1) earlier and greater degeneration of the skin in the right forelimb region, and (2) greater size and strength of the right limb. As these results are directly opposed to the observations of Gudernatsch, Dickerson and myself on five species of frog tadpoles, it can only be supposed that there is a species difference, and that the factors mentioned by Barfurth are strong enough to bring about a majority of righthanders in this particular species. It would be of interest to see whether in this species also the uniform lefthanded condition could be produced by thyroid treatment.

The explanation of the results after thymus treatment is somewhat more difficult and uncertain. It is probable that a shift toward righthandedness is here indicated. The ratio may be 1 : 1, although on account of the small number of animals observed in the three-limb stage, this is by no means a certainty. At any rate, the lessening of the lefthanded majority means that the asymmetrical position of the spiracle becomes of much less importance as a factor in determining the first forelimb to appear.

Desiccated thymus extract is a food rich in nutritive value, and therefore favorable to growth. Gudernatsch ('14) noted its growth-promoting effect upon tadpoles and ascribed it to the endocrine secretion of the thymus. Uhlenhuth ('17) though he combats Gudernatsch's idea as to the growth effect being due to an endocrine secretion contained in the thymus extract, states that it is a very rich and nutritious food and therefore quite favorable to growth. The writer has also observed that it is particularly favorable to limb growth. In one batch of partly-grown thymus-treated green frog tadpoles the small hind limbs

became quite red and vascular and grew rapidly, almost reminding one of the effect of thyroid extract. The writer became suspicious of the thymus extract used and had it analyzed for the presence of iodine.[5] The analysis gave negative results. The later history of the tadpoles showed that only growth in size of the larval structures was being stimulated, and not differentiation. There was no acceleration in skin degeneration of the forelimb region, except that caused secondarily by pressure of the growing forelimb. There was likewise no reduction in the tail, but on the contrary growth. With thymus treatment limb growth appears to proceed relatively faster than the general process of body differentiation. As a result, the forelimbs enclosed beneath the skin reach a comparative size and strength, such that they become the important factor in determining the time of eruption. With increasing limb size the spiracle becomes less important since the forelimb cannot be pushed through it without complementary skin degeneration. Since size of forelimb and amount of skin degeneration on the two sides are about equal in the species under observation a more equal ratio of righthandedness to lefthandedness results.

The writer does not wish here to enter the controversy as to whether or not the thymus extract has a specific endocrine content. Its effect upon symmetry in forelimb eruption in tadpoles seems to be best explained on the grounds given above; *i.e.*, its unquestioned value as a highly nutritious and therefore growth-promoting food. It is not necessary to assume a specific endocrine effect. This much, however, may be added. Thymus gland is largely lymphoid tissue. In the light of Carrel's work showing the growth-promoting effect of leucocytic secretions or "trephones" ('24), and the confirmatory observations of Jordan and Speidel ('23) on lymphocytes in rapidly growing regions in tadpole metamorphosis, it would seem probable that growth-promoting substances (trephones) of lymphocyte origin would be present in thymus extract.

In conclusion, it may be pointed out that these observations and their interpretation, though of little importance in themselves, suggest the possibility of the following principle operating

[5] The analysis was made by Mr. T. F. Otto, of the University of Virginia Medical School.

in any vertebrate animal in process of development: A change in the normal balance of thyroid secretion may lead to a change in the symmetrical development. Stockard ('23) has emphasized the general importance of thyroid secretion in the development of man and mammals and its part in determining the production of definite types. These results on forelimb eruption in tadpoles indicate that thyroid secretion may be of some importance also in influencing symmetrical development. A vertebrate animal, though designated as bilaterally symmetrical, is, of course, asymmetrical in many respects, *e.g.*, the visceral pattern, much of the vascular system, etc. Given an original asymmetrical condition it is possible that the thyroid may exert its effect upon the two sides of the body in a differential way.

Summary.

The bilaterally symmetrical development of the frog larva is affected in a definite way by experimental hyperthyroidism. Normally in tadpole metamorphosis the left forelimb erupts first in about 70 per cent. of cases. With thyroid-accelerated metamorphosis of half-grown tadpoles practically 100 per cent. will put out the left forelimb first. Of 133 thyroid-treated animals of this kind every one protruded the left forelimb first. In the case of full-grown tadpoles already near the time of forelimb eruption, thyroid treatment may be followed during the first two days by some prior right forelimb eruptions; thereafter prior left forelimb eruption obtains. In other words, an original bias of an animal toward prior right forelimb eruption may not be changed by thyroid administration within two days.

This effect of the thyroid on symmetrical development is explicable in terms of the original asymmetrical pattern of the respiratory apparatus (*i. e.*, sinistrally located spiracle) coupled with the close anatomical relation of the forelimb to this apparatus.

Thymus treatment brings about a larger percentage of prior right forelimb eruptions, thus reducing somewhat the normal majority in favor of prior left forelimb eruption.

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CELL SIZE AND METABOLIC ACTIVITY IN AMPHIBIA.

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I. Introduction.

The fact that the mass of a body increases as the cube of the linear dimension, while the surface increases as the square, has long been recognized as of importance in biology. Leuckart (1852) uses it to explain the necessity for increased surface as an organism becomes larger, a necessity which is met in animals by inpushings and the develompent of a distributing system; in plants by outgrowths. Herbert Spencer (1873) says: "Why has the individual a growth limit? . . . In similarly shaped bodies, the masses vary as the cube of the dimensions, whereas the strengths vary as the square of the dimensions." He applies this idea to an individual whose height doubles in a given growth period; the mass has been multiplied by eight, but the strengths of muscles and bones, being proportional to their cross section, are multiplied by only four. The absorbing surface is also multiplied by four, while the mass to be nourished by the material absorbed is multiplied by eight. It is only a step farther to apply the same idea to cell size. The writer was unable to learn who first did this.

Since the absorbing surface of a cell increases only twice as fast as the radius, while the mass to be nourished by absorbed material increases four times as fast, it follows that there is a definite size limit to cell growth; and further it is evident that the absolute size attainable by any given cell is inversely proportional to its rate of consuming the material absorbed. In other words, a sluggish cell may absorb the relatively small amount of material needed for its activity through a smaller surface than a more active cell, with its greater requirements of material; or the sluggish cell could grow to a larger size than the active one and still get sufficient material through its surface. If this is true, then one may reasonably expect a sluggish animal to have larger cells, while a more active animal would require smaller cells.

The research described in the present paper was started with the idea of obtaining some experimental data which might indicate whether the assumption stated above is true, and therefore whether the size of cells might be of fundamental importance in the activities of an animal. The Amphibia were chosen for experimental material because they have quite large cells which can be measured with less error than smaller cells, and because the Amphibia are known to vary quite widely in both cell size and activity.

Gulliver (1875) published measurements on the blood corpuscles of 650 species of Vertebrata; including 3 Cyclostomata, 11 Elasmobranchii, 75 Pisces, 17 Amphibia, 38 Reptilia, 265 Aves, and 241 Mammalia. Perusal of his figures with a consideration of the relative degree of activity of the various animals indicates a general agreement with that to be expected if cell size does vary inversely with activity, but there are numerous exceptions. Most of these exceptions can be explained on the basis of the size of the animal, for Gulliver points out that, within a limited group, the size of the red blood corpuscles increases with increasing weight of the members of the different species considered. That activity may be of importance in connection with cell size is indicated by the fact that, among the Mammalia, the smallest corpuscles are found in the deer family, the largest in the elephant, porpoise, anteater, and sloth and near the average size among the Carnivora. Among the Cheiroptera the

fruit-eating bats have distinctly larger corpuscles than the insect eaters. Among the birds the largest corpuscles are found among the Cursores, and the smallest among the insect-eating passeriform birds. Among the reptiles the Chelonia have distinctly larger corpuscles than the Sauria. The corpuscles are much larger in the Caudata than in the Salientia, and larger in the Elasmobranchii than in the Pisces.

Other workers who have given measurements of red blood cells are Weckler (1863), Malassez (1872), Formad (1888), Wormley (1888), and Forrest (1913). Forrest and Malassez also made counts. These run in inverse ratio to the size, although there are exceptions. A comparison of the measurements of amphibian corpuscles by different workers will be found later. Reichert and Brown (1909) review the work which has been done on red blood cell size and state that attempts to correlate the size of these cells with the rapidity of the animals' movements are founded on insufficient or erroneous data.

Hartmann (1919*a*) shows that the chloroplasts in developing *Elodea* leaves are smaller and more numerous in plants grown at higher temperatures, as contrasted with the larger and less numerous chloroplasts in plants grown at lower temperatures. Since the metabolism of the leaves is certainly speeded up with increased temperature, this observation falls well in line with the idea that a high rate of activity is associated with small size.

Chambers (1908) shows that there is considerable variation in the size of the eggs of *Rana esculenta* and *R. temporaria*, that the larger eggs develop a little more rapidly than the smaller ones, and that there is a much higher percentage of mortality among small than among large eggs, especially when grown at higher temperatures. He shows that the size of the cells in the frog varies with the size of the egg from which the frog developed; and that eggs allowed to develop at higher temperatures invariably yield smaller frogs with smaller cells than those developed at lower temperatures (size taken at time of metamorphosis or earlier). Tadpoles in crowded cultures are smaller than those with more room, but this does not affect the size of the cells. Morgan (1904) worked on dwarf frog eggs which had only about half the volume of the normal eggs, and showed that the cells in the developing dwarf embryos tend to remain smaller

than normal. Berezowski (1910) worked on the size of the intestinal cells of the white mouse during development, and shows that these cells become larger as the animal grows.

Krogh (1916) gives comparative tables on basal metabolism as it has been worked out by various workers on different animals. These figures indicate that there is a general agreement between activity as measured by basal metabolism, and cell size as measured on the red blood cells by Gulliver (1875), in inverse ratio. Figures given by different workers on metabolism vary quite widely, and this is true to an extreme degree of Amphibia. For instance Regnault and Reiset (quoted in Morat and Doyon, 1900) found that 0.063 mg. of CO_2 was eliminated per gram of frog per hour, while Krogh (1916) gives a figure which corresponds to 0.3686 mg. of CO_2 per gram per hour.

II. Material and Methods.

As many different species of Amphibia were used as it was possible to obtain. Activity was measured in terms of carbon dioxide output. This was measured by fixing the gas as a precipitate of barium carbonate in a barium hydroxide solution. In detail the method consisted in sucking air by means of a filter pump through one 8-inch tube of concentrated potassium hydroxide, two 8-inch tubes of soda lime and a gas washing bottle containing strong barium hydroxide. This series was to remove the carbon dioxide from the atmospheric air. The stream of air then passed into a respiration chamber containing the animal. Even when air breathing animals were used some water was always placed in the bottom of this chamber to keep the skin of the animal moist. From the respiration chamber the air current passed through two or three gas-washing bottles containing a carefully measured amount of standardized barium hydroxide. Special care was taken to see that the air was broken up into fine bubbles as it passed through these bottles. To accomplish this the end of the inlet tube was drawn out into two fine points. The bulb type of bubbler was found unstaisfactory because it was too easily broken in the numerous manipulations incident to making a long series of determinations; and because a finer stream of bubbles could be obtained by the method described. Suction tubing was used in making connections, and special precautions were taken to avoid leaks.

Standard solutions were made by preparing a stock solution of N/10 oxalic acid by weight, N/10 barium hydroxide standardized against this, and N/10 hydrochloric acid standardized against the barium hydroxide. Phenolphthalein was used as an indicator in preparing the standards and in the actual determinations. A preliminary aëration was run with the animal in the respiration jar, but without the collecting bottles, for one hour. The collecting bottles were then placed in the series, and aëration carried on for a measured length of time, 8–24 hours. At the end of this aëration the collecting jars were removed and the excess of hydroxide titrated immediately by means of N/10 hydrochloric acid. The amount of hydroxide used by the carbon dioxide was thus obtained by difference, and the amount of carbon dioxide collected computed as CO_2 per gram body weight per hour. All determinations were made on starving animals and at room temperature, which varied between 20 and 23° C.

Truog (1915) describes a method of determining carbon dioxide by passing air through a tower containing barium hydroxide and glass beads. This method would be more accurate than the one used here, but the method was unknown to the writer at the time when the experiments described in this paper were undertaken. It is felt that the method used here yields results of comparative value, which is all that is needed. Truog shows that the barium carbonate present with the hydroxide does not hinder accurate titration, and that the barium hydroxide method of determining carbon dioxide is very accurate.

Red blood corpuscles were used for measuring cell size. Most of the animals used were those on which carbon dioxide determinations had already been made. The animal was killed either by pithing or with chloroform, and blood taken either from the heart by means of a syringe or from the tail vein. Thin smears were made on slides, dried in the air and stained with Wright's stain. In many cases blood counts on both red and white corpuscles were made, and tissues fixed in Bouin's fluid for section later in order that other cells might be measured. The blood counts and tissue cell measurements are not included in the present paper. The latter agree reasonably well with the results given for red blood cells, while blood counts are found to vary widely with the physiological condition of the animal. Of course in general animals with larger cells have smaller numbers.

Red blood cells were measured by means of a scale which was so constructed that each division on the scale corresponds to one micron in the oil immersion field (B. & L. 1.9 mm. obj.) with 10x ocular and 160 mm. tube length, when the scale is placed on the table beside the microscope and viewed through a camera lucida with mirror set at an angle of 45° and the arm length set at 103 mm. With the aid of this scale the dimensions of red blood cells were measured, 50 cells being measured from each slide, and from one to five slides being used for each animal studied. From the average length and width thus obtained the surface of the average corpuscle for each animal was computed, assuming no thickness, from the formula $\pi LW/2$ where L is the maximum and W the minimum diameter. This formula follows from the formula for a regular ellipse, πab, where a and b are the long and short radii. The surface is used as the significant figure rather than the length and width because all corpuscles are not the same shape, length and width have different ratios, and therefore the dimensions do not give a direct index of size.

III. Results for the Different Species of Amphibia.

Table I. gives the results for the various species used. Where a space is left blank, no data were obtained on the particular point concerned. For instance it will be noticed that sex is not given in a number of cases, and in the same cases usually no blood cell measurements are given. In these cases the animal died and it was not considered safe to make blood cell measurements on such animals in which post mortem changes had had time to occur. Therefore they were not autopsied at all, and thus no data were obtained on sex. In several species it was impossible to obtain samples for carbon dioxide determinations, although one or several had already been used for blood smears.

A short description of the material and results for each species is given below. The species are taken in the same order as in the table; that of red blood cell size.

Amphiuma means (Gordon).—This species has the largest corpuscles known for any amphibian. Three adult specimens and one young were obtained from New Orleans. Measurements of carbon dioxide output were made at intervals over a period of two weeks. The animals showed evidence by their

Table I.

Cell Size and Carbon Dioxide Production for the Different Individuals Used in Each Species Studied.

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Cm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Amphiuma means.							
F 1......	large			50	62.8	35.42	3.494
F 2......	41.4	0.0920	3	150	60.9	34.8	3.329
3......	1,243	0.02136	3	50	62.18	36.8	3.594
4......	1,270	0.02146	3	50	62.06	37.42	3,648
5......	1,500	0.02125	2	50	61.0	39.04	3.741
Necturus maculosus.							
F 2.....	154	0.0420	5	250	53.78	30.8	2,602
M 3.....	194	0.0413	5	250	57.45	30.62	2,763
F 4.....	126	0.05285	16	250	54.568	27.496	2.357
M 5.....	130	0.0548	4	250	50.152	26.012	2,049
M 6.....	115	0.0532	10	150	52.386	26.46	2,177
M 7......	126	0.05576	13	25	55.52	29.24	2.550
F 8......	57.6	0.0922	12	250	51.144	30.984	2.489
M 9......	55.7	0.1069	13				
M 10.....	86	0.0883	1	50	54.44	30.54	2,612
11.....	47	0.1399	2				
F 12.....	75	0.0915	1				
F 13......	93.5	0.0842	2				
M 14......	92	0.0753	1	50	54.04	25.7	2,187
15......	74	0.1051	1				
F 16......	44.3	0.1208	3	50	50.38	31.66	2.505
F 17......	64	0.1192	2	50	53.72	25.28	2,133
18......	194	0.0599	1				
Cryptobranchus alleganiensis.							
1......				50	41.12	23.16	1,496
Diemyctylus viridescens.							
1......				50	29.64	17.76	827
Rana catesbiana							
F 1......	455	0.0629	4	250	25.2	13.032	516
F 2......	604	0.0527	3	250	27.324	13.444	578
F 3......	714	0.05257	4	50	25.58	12.86	517
F 4......	510	0.0929	5	50	25.62	16.18	651
M 5......	361	0.0671	3	250	26.836	13.176	555
6......	503	0.0584	3				
M 7......	388	0.0704	3	50	25.38	14.14	564
8......	492	0.0627	3				
M 12......	507	0.0876	3	50	24.48	13.84	532
F 13......	411	0.0699	3	100	26.07	16.82	702
14......	361	0.0787	3				
F 15......	628	0.0843	3	50	23.86	14.16	531

Table I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Rana clamitans.							
1				530	22.072	12.498	433
F 2	19	0.1794	4	250	23.236	12.22	446
M 3	7.1	0.1359	4	100	23.64	11.03	409
Rana pipiens.							
F 1	43.4	0.1386	9	100	19.53	12.56	385
M 2	25.4	0.1422	17	150	20.37	11.32	362
F 3	45.8	0.1768	7	250	21.078	14.192	470
M 4	27.0	0.2026	5	250	22.96	13.644	492
M 5	30.5	0.1762	13	50	19.86	13.78	421
M 6	33.6	0.1394	10	50	21.24	12.96	432
F 7	59.0	0.1795	3				
F 8	43.5	0.1931	6	50	18.46	13.42	389
F 12	54.7	0.1145	2				
F 13	52.6	0.1314	1				
M 14	32.7	0.1103	1				
F 15	40.8	0.1765	5				
M 16	28.9	0.2002	7				
M 17	14.4	0.2037	1				
Rana palustris.							
1				100	20.47	13.94	448
F 2	5.7	0.2742	5	200	20.11	12.705	401
M 3	3.6	0.2918	8	250	19.12	12.16	365
F 4	3.7	0.2531	9	250	20.384	12.152	389
F 5	3.9	0.1710	2	100	21.58	12.44	422
6–17	36	0.1603	2				
18				50	20.7	13.96	454
M 19				150	19.13	12.47	375
Hyla pickeringii.							
1				50	19.44	11.2	342
Acris gryllus.							
1				50	17.82	11.18	313
2				50	17.7	10.6	295

TABLE I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Chorophilus nigritus.							
1......				50	17.96	10.04	283
Bufo americanus.							
F 2......	11	0.1831	5	250	16.044	9.684	244
3......	1.9	0.2069	1				

feces of having eaten recently. The baby specimen was killed and blood smears made immediately at the termination of the carbon dioxide output measurements; the adults were bled from the tail vein after six weeks.

Necturus maculosus (Rafinesque).—All specimens except the first two used were collected from Lake Mendota at Madison. They were kept in cold running water without food until used. From the time of the first determination on an individual until it was killed or died it was kept at room temperature. The first two specimens were received from dealers, and their source is unknown. In this species starvation was carried on over a long period of time, and its effect on metabolism studied. Individuals of widely varying weights were used and the effect of weight on metabolism noted. The results of these experiments will be described later.

Cryptobranchus alleganiensis (Daudin).—The single specimen used was a laboratory specimen of unknown source from which blood smears were made. No individuals were available when needed for metabolism tests on account of the cold weather.

Diemyctylus viridescens (Rafinesque).—One specimen was used for blood smears. It was collected in New York state during the summer, and blood smears were made in the fall. Probably the animal had not eaten in the meantime.

Rana catesbiana (Shaw).—Twelve specimens were obtained from New Orleans. Specimen No. 1 had had the lower jaw broken at some previous time, and it had healed in such a manner

that eating was impossible and the animal was in an extreme state of starvation. The other specimens showed signs of having recently eaten, and were well fed. They were kept at a temperature of about 12° C. when not being used for carbon dioxide output tests. Attention is called to specimen No. 4, which shows an unusually high carbon dioxide output. It is not included in the general average for the species in Table VIII. The detailed record for this animal follows: 2/10—0.0653, 2/29—0.0639, 3/20—0.1208, 4/18—0.1037, 4/20—0.1110 (dates of determinations and carbon dioxide output figures). No explanation is found for this strange behavior; starting with approximately a normal carbon dioxide output value, jumping to twice the normal, and remaining there. That the phenomenon is not due to an acute infection is indicated by the fact that the animal appeared normal on autopsy a month after the final test.

Rana clamitans (Latreille).—Two specimens were collected near Madison and kept at room temperature until used three months later. They had no food during this time. Blood smears were made from No. 1 immediately after it was collected. The low value for carbon dioxide production with the small animal is probably due to the extreme emaciation of this specimen. Neither cell size nor activity results differ essentially from those of the following species.

Rana pipiens (Shreber).—The specimens used were from several shipments from supply houses. Some of these animals were starved at room temperature for a long time, and the effects of lack of food on metabolism were noted. No appreciable decrease in metabolism resulted until the animal had reached an extreme state of starvation. No. 2 lost 47 per cent. of his body weight before any marked drop in carbon dioxide output per gram of body weight was obtained. Several specimens died of disease. The results of the study of metabolism during the course of the disease will be described later.

Rana palustris (Le Conte).—The specimens used were collected near Madison in the late fall. They were kept at room temperature and used for carbon dioxide output tests over a period of two months. At the end of this time they were all very weak from starvation. Nos. 1, 2, and 3 were killed while still in good condition. No. 5 and Nos. 6–17 were tested when near death

from starvation. No. 18 was starved, while No. 19 was used for blood smears within a few days after being collected.

Hyla pickeringii (Holbrook).—One specimen was collected and blood smears made before the carbon dioxide tests had been started. None were available while tests on metabolism were being made.

Acris gryllus (Le Conte).—Two specimens were collected from the field and blood smears made. Time was lacking to make carbon dioxide output tests.

Chorophilus nigritus (Le Conte).—See note on *Hyla pickeringii*.

Bufo americanus (Le Conte).—Two specimens were collected and starved at room temperature three months before using. No. 3 died of starvation after only one test for carbon dioxide had been made, and blood could not be obtained for smears.

IV. Controls.

A. *Cell Size.*

1. Blood corpuscles may shrink as smears dry. However the same methods were used on all specimens, so that the results should be comparable. That there is a certain amount of differential shrinking due to differences in thickness of smear is suggested by comparing measurements from different slides made from the same individual. For instance the five slides of blood from Rana clamitans No. 2 gave the following series of averages of 50 corpuscles from each slide: 23.98 x 13.02, 22.92 x 11.66, 23.32 x 12.08, 23.1 x 12.32 and 22.86 x 12.02. Some of this variation may have been caused by differences in the corpuscles which happened to be measured, but most of it was probably due to the nature of the smear. With the slide which averaged 23.98 x 13.02 the first 25 corpuscles measured 23.84 x 13.12, while the last 25 measured 24.12 x 12.92. The method used is that of Gulliver (1875) except for the fact that he does not appear to have stained his smears. Georgopolus (1906) states that dry preparations are unreliable because the size of the cells is likely to vary with the thickness of the film, and states his preference for the wet method. This consists in placing a small drop of fresh blood on a clean slide and quickly placing on a cover. The corpuscles are then measured immediately. This method has been found useless for Amphibia because the corpuscles are distorted by the

treatment. For the smaller mammalian corpuscles it is an excellent method. The one attempt which was made to use the wet method gave sizes which checked fairly well with measurements from dried smears. It was found to be difficult to use an oil immersion objective on a wet preparation, and it was difficult to find corpuscles which were not distorted.

For convenience of reference for the reader, and to show how the present measurements check with those given by other workers, Table II. has been prepared to show the sizes of amphibian corpuscles according to the measurements of other workers. The writer's measurements are not included here because they are mostly on different species, the table would therefore be much longer, and the measurements are found elsewhere in this paper.

Table II.

Measurements on Amphibian Red Blood Corpuscles as Made by Different Workers.

Species.	Gulliver.	Weckler.	Wormley.	Forrest.	Morat and Doyon.
Amphiuma tridactylum (means)	69.9 x41.3		70.9x40.9		
Proteus angineus	63.5 x34.94	58.2x33.7			58.0x35.0
Siren lacertina	60.47x33.42				
Cryptobranchus japonicus	56.45x31.75				
Cryptobranchus alleganiensis	45.11x25.4				
Siredon humboldtii	44.8 x25.4				
Lissotriton punctatus	31.75x19.84				
Salamandra				37.8x23.8	
Triton bibronii	29.95x19.84	29.3x19.5		29.3x19.5	29.3x19.5
Triton cristatus	29.95x19.84				
Rana		22.3x15.7	23.3x14.1	22.3x15.7	22.3x15.7
Rana esculenta	25.4 x17.58				
Rana temporaria	22.92x13.95				
Bufo		30.2x18.2		21.8x15.9	
Bufo vulgaris	24.35x12.7				
Bufo clamita	19.05x13.4				

2. It is possible that some of the animals studied by the writer show abnormally small corpuscles on account of extreme starvation. *Necturus* No. 6 and *Rana pipiens* No. 2 are cases with extreme starvation and small cells. However, *Necturus* Nos. 7 and 8 and *Rana pipiens* No. 5 also underwent extreme starvation,

and show relatively large cells. *Rana palustris* No. 19 was collected from the field and blood smears made immediately, yet this specimen shows the smallest corpuscles measured for this species. On the other hand *Rana palustris* No. 18 shows the largest corpuscles of the species, and was used when in an extreme state of starvation. There seems to be no correlation between cell size and degree of starvation, as judged by the size of the red blood cells.

B. Carbon Dioxide Determination.

1. The apparatus used has two inherent defects. These are not considered to be of sufficient importance to affect the results for the relatively large amounts of carbon dioxide measured. They are, first that rubber tubing was used for all connections, and second that soft glass bottles were used for collecting jars. Rubber tubing has been shown to have a selective absorption for carbon dioxide, but this should not be important considering the short lengths of tubing used, the rapidity of the air stream, and the relatively large amounts of carbon dioxide collected.

2. To learn whether the traps to remove carbon dioxide from the air before it entered the respiration chamber were taking out all the gas, a gas washing bottle containing a carefully measured amount of standard barium hydroxide was placed between the traps and the respiration chambers. 22 hours of rapid aëration yielded 5.06 mg. of CO_2, or 0.23 mg. per hour.

3. To learn whether some of the expired CO_2 was getting by the collecting jars, a barium hydroxide bottle as in the previous case was placed between the collecting jars and the pump. A rapid stream of air passing through two respiration jars and two sets of collecting jars (in parallel; one for *Necturus*, one for *Rana pipiens*), was sucked through this jar for 26 hours. 28.6 mg. of CO_2 were collected, or 1.1 mg. per hour. Subtracting from this figure the amount introduced into the jars with the inhaled air, it appears that 0.87 mg. per hour of CO_2 was being lost from the system. The animals in the jars weighed 93 grams, so that the loss is 0.00936 mg. per hour per gram weight of the animals. This is about an 8 per cent. loss for *Necturus* and about 6 per cent. for the frog. For the larger animals an additional collecting jar was used. This of course tended to keep down the loss.

4. Leaks were practically eliminated. It was possible to develop a strong negative pressure in the jars, close all valves, and allow the apparatus to stand for several hours with no appreciable diminution of pressure.

5. The negative pressure in the respiration jars averaged about 2 cm. of mercury. This factor was practically constant for the entire course of the experiments.

6. Measurements of the rate of flow with the air current moving at as near the average rate as was possible to judge yielded two minutes for each liter of air. This stream was divided between a jar of four liters capacity (used for *Necturus*, *Rana catesbiana* and *Amphiuma*) and one of one liter capacity (used for the smaller animals). Tests on each jar separately showed that the air in the large jar was being changed every eleven minutes, while that in the small jar was changed every five minutes. This should be sufficient speed in each case to keep the atmosphere around the animal relatively free of carbon dioxide. Attempts to cut down the rate through the small jar to more nearly equal that of the larger jar were unsuccessful because it was found that, with too slow a rate, the holes in the bubblers in the gas collecting jars became clogged with precipitate of barium carbonate and the aëration stopped.

7. Determinations of the dissolved carbon dioxide in the water surrounding the animal at the end of the preliminary aëration and again at the end of the final aëration yielded approximately the same figure in each case. For instance, while a carbon dioxide test was being made on a bull frog, no titratable CO_2 was found in the water used in the jar, 5.5 mg. CO_2 per 100 cc. were present in the water at the end of the preliminary aëration, and the same figure at the end of the final aëration.

8. Several blanks were run with the regular amount of water in the respiration jars, but without animals, and one with a completely empty jar. The results of these trials are shown in Table III.

For all the above tests 250 cc. of N/10 barium hydroxide were measured into two gas-washing bottles, and the bottles filled to capacity (300 cc.) with distilled water. This same procedure was followed in filling the jars for the regular carbon dioxide output tests. For numbers 7 and 8 solutions were used which

were not quite standardized. Therefore No. 8 is of value only in comparison with No. 7. Subtracting the two it is found that the 14 hours of aëration resulted in the accumulation of 11 mg. of CO_2, or 0.785 mg. per hour. Comparing this with No. 6 it is seen that 1.275 mg. per hour resulted from the presence of water in the respiration jar in the aëration numbered 6. The source of this CO_2 is the dissolved bicarbonate of the water, which gradually liberates carbon dioxide when CO_2 free air is bubbled through it. This is not a factor when an animal is in the jar, as is shown by the determination recorded above of the amount of dissolved CO_2 in the water surrounding a respiring animal; sufficient to prevent liberation of the gas from the bicarbonate. Trials Nos. 1, 2, 3 and 5 indicate that this combined CO_2 comes out quite slowly. No. 4 represents the degree of accuracy which can be expected from the titration. This figure represents 0.8 cc. of N/10 barium hydroxide in 250 cc.; and thus is an error of 0.32 per cent.

TABLE III.

BLANK TESTS ON CARBON DIOXIDE.

Trial No.	Hrs. of Aëration.	Mg. CO_2 Collected.	Mg. CO_2 per Hour.	Remarks.
1	5	5.02	1.012	Small jar. One liter capacity.
2	9	5.72	0.635	Small jar.
3	14	8.36	0.597	Small jar.
4	0	1.76	—	Treated as for aeration, but titrated immediately.
5	27	11.98	0.444	Small jar.
6	24	49.5	2.06	Large jar. Four liters cap.
7	14 (24)	18.48	0.486	No water. Large jar, 14 hrs. aëration plus 24 hrs. standing.
8	(38)	7.48	0.197	Parallel with No. 7. No aëration; standing.

It appears, then, that the carbon dioxide collected in the blank aërations is from three sources: (1) that due to the liberation of the bound CO_2 of the water, (2) that caused by residual CO_2 in the atmosphere of the respiration chamber at the end of the preliminary aëration, and (3) that resulting from CO_2 getting through the air washing system before the air enters the respiration chamber. Of these only the last is of importance in producing error, the others being eliminated by the presence of a

respiring animal in the jar. This factor has already been shown to be overcompensated by the loss resulting from incomplete absorption of the carbon dioxide by the barium hydroxide. Inasmuch as the first two factors are important, they would tend to neutralize this loss. It is therefore concluded that the method is sufficiently accurate to allow for making comparative carbon dioxide output determinations on the animals used.

Indirect evidence that the factors discussed above are not important in producing error is obtained by comparing the results obtained on different animals. Errors caused by the passing through of excess carbon dioxide would tend to increase the apparent result for small animals more than for larger ones, while for small animals the error resulting from the loss of carbon dioxide that was not absorbed would tend to be minimized. With these ideas in mind, if one looks at the results for *Rana palustris* (Table I.) it is apparent that Nos. 6–17 have within the limits of variation the same carbon dioxide output result as No. 5, although the latter has twelve times the chance of being thrown off by the errors as have the former. Again *Bufo americanus* shows only slightly higher results for a 1.9 gm. individual as for an 11 gram one, and *Rana clamitans* shows a lower result for the lighter animal.

V. Complicating Factors.

A. Cell Size.

1. If the figures presented in this paper are compared with those obtained by other workers, it will be found that they run decidedly low. Measurements are presented for the same species only in the case of *Amphiuma* and *Cryptobranchus*, but the indications are in the same direction for the frogs and toads, in which different species have been used here than those used by other workers. There are two possible causes for this difference; first that the writer has obtained shrinkage of the corpuscles, as previously pointed out, and second that the measuring technique used is faulty. Several attempts have been made to check the latter point. The scale used has been repeatedly compared with a Zeiss stage micrometer, and found to be accurate. By means of an ocular filar micrometer the widths of the different 10 micron divisions of the Zeiss stage micrometer have been measured.

TABLE IV.

VARIATION IN RED BLOOD CELL SIZE IN DIFFERENT SPECIES OF AMPHIBIA.

Number of corpuscles measured corresponding to each size.

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Cero.*		*Bufo.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
83	1						34			1															
77	2						33	1		3															
76	1						32	7		6															
74	1						31	11		16															
73	2						30	10		38															
72	3						29	9		109		3													
71	6						28	5		120		5													
70	5		2				27	4		149		22		4											
69	7						26	3		230		40		4		1									
68	8		5				25	3		214		77		30		4									
67	18		7				24			97		140		82		16		1							
66	17		7				23			62		155		111		57									
65	24		15				22			37		121		150		98		3							
64	14		12				21			14	1	104		143		142		8							
63	37		16				20		5	7	1	86		162		305		8		7		7			
62	32		33				19		10	1	7	45		122		202		18		18		12		3	
61	27		34				18		16		33	24		63		128		10		37		14		37	
60	32		62				17		10		51	8		16	3	51		2		22		9		53	
59	19		52				16		5		46	1	2	4	13	6	1			14		5		67	
58	21		85				15		3		148		29		132		21			2		3		65	
57	13		99				14		1		292		105		296		112							18	
56	15		98				13				314		186		233		323		3		3			6	
55	16		139				12				130		231		124		379		15		28		2	1	1
54	7		107				11				44		148		58		144		22		32		8		30
53	3		111				10				24		78		33		20		9		30		31		121
52	2		167				9				2		1		11				1		8		8		85
51	5		122				8																1		13

TABLE IV.—*Continued.*

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra.	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Coro.*		*Bufo.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
50	5		164																						
49			95																						
48	1		74																						
47			74																						
46			49		1																				
45	1	1	31		4																				
44	1	5	9		3																				
43		4	9		8																				
42		7	2		8																				
41		13	1		5																				
40		28	1	1	7																				
39		21	1	6	4																				
38		37		9	2																				
37		31		21	3																				
36		52		33	3																				
35		47		57	1																				
34		28		61																					
33		28		110		1																			
32		20		131																					
31		14		151																					
30		7		176																					
29		4		161																					
28		2		149																					
27				138																					
26				123		3																			
25				138		3																			
24				95		10																			
23				63		8																			
22				36		10																			
21				20		7																			
20				6		3																			
19				1		1																			
Totals	350		1700		50		Totals	50		1100		830		900		1000		50		100		50		250	

It is found that these divisions vary in width from 5.8 per cent. below the mean to 5.6 per cent. above it. With such wide variations between the different divisions of the micrometer, the question naturally arises as to whether the entire micrometer may not be inaccurate. As a third check on the method the dimensions of corpuscles as measured by means of the scale were compared with the dimensions of the same corpuscles as measured by means of the filar micrometer. It was found that the scale is less accurate for individual corpuscles, because it is impossible to measure with it to an accuracy of less than one micron, but the average of a series of measurements by the two methods gave closely parallel results.

2. In the Amphibia the size of individual red blood cells in the same animal varies so widely that averages only partly represent the peculiarities of the different species. In many cases

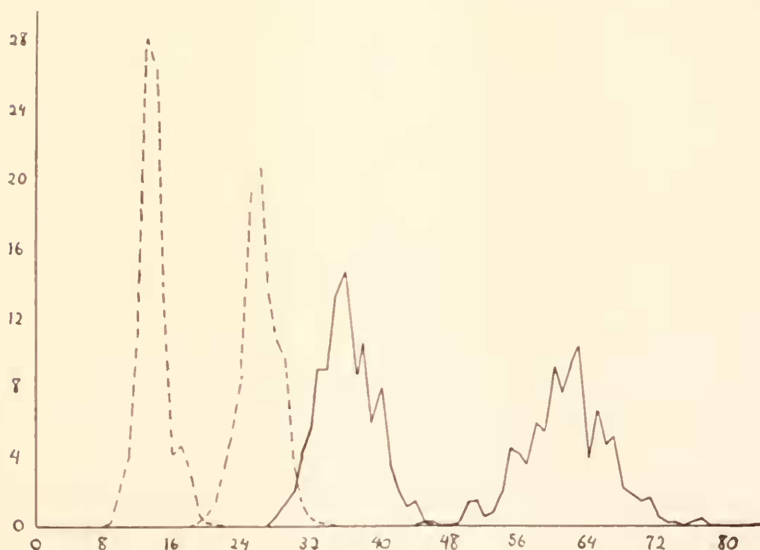

CHART 1*a*. Variation in red blood cell size for *Amphiuma* (solid line) and *Rana catesbiana* (broken line). Abscissa, dimensions in micra; ordinate, percentage of total corpuscles measured. Two curves are given for each species, one representing the long diameters of corpuscles, and the other the short diameters.

the range of variation in size is just as characteristic as the average size. For this reason Table IV. has been prepared to show the range of size variation in each species. In many cases

there are either two maxima or a sustained maximum. This results from the fact that the maximum for different individuals of the same species varies. It is just this variation between individuals which complicates comparative results on cell size, especially between closely related species. It is not associated with the metabolic activity of the animal, sex, or any other factor which can at present be indicated. Reference to Chart 1 will aid in understanding the variations here discussed.

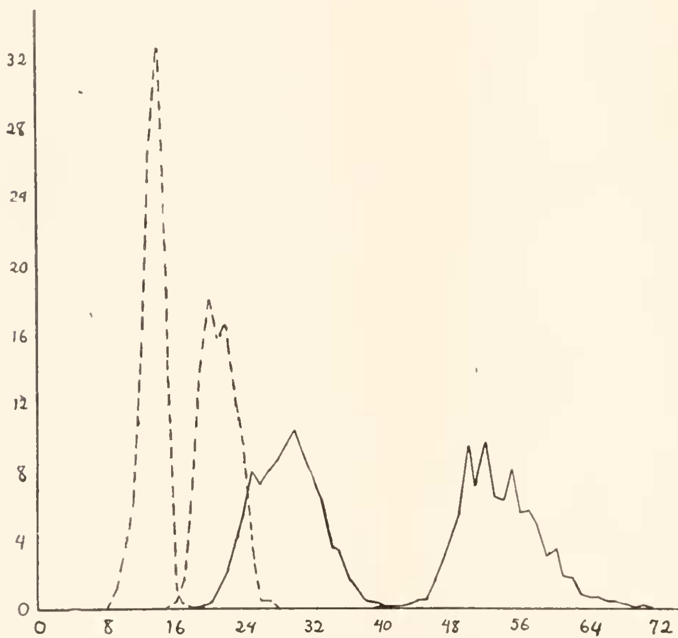

CHART 1*b*. Variation in red blood cell size for *Necturus* (solid line) and *Rana pipiens* (broken line). Explanation as for Chart 1*a*.

B. Carbon Dioxide Production.

1. The weight of the animal is the primary factor which complicates the results on carbon dioxide production. The general fact is that the carbon dioxide output as measured by unit weight increases as the weight decreases. Reference to the results for *Necturus* will emphasize this fact. For convenience these are listed in Table V., the individuals being taken in order of their weight.

TABLE V.

Necturi ARRANGED ACCORDING TO WEIGHT TO SHOW VARIATION OF CARBON DIOXIDE PRODUCTION WITH VARIATION IN WEIGHT.

No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.
3	194	0.0413	6	115	0.0532	17	64	0.1192
18	194	0.0599	13	93.5	0.0842	8	57.6	0.0922
2	154	0.0420	14	92	0.0753	9	55.7	0.1069
5	130	0.0548	10	86	0.0883	11	47	0.1399
4	126	0.0528	12	75	0.0915	16	44.3	0.1208
7	126	0.0557	15	74	0.1051			

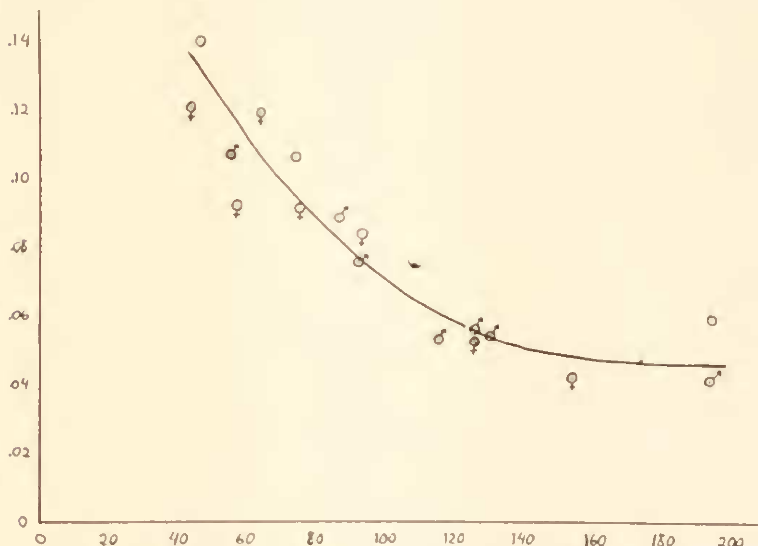

CHART 2. Influence of weight on carbon dioxide output in *Necturus*. Abscissa, weight in grams; ordinate, CO_2 in milligrams per gram of body weight per hour. ♀ = females, ♂ = males, ⊙ = sex not determined.

The general trend is evident from Table V., but exceptions are also evident. Nos. 18, 13, 15, 17, and 11 are higher than would be expected from their weight, while Nos. 2, 6, 14, and 8 are lower. The high group contains only animals on which one or two carbon dioxide output tests could be made before the animal died. The cause of death in all these animals was probably starvation, there being no pathological symptoms as far as could be determined. A more detailed discussion of starvation as a complicating factor will be found below. It is more difficult to

understand the meaning of the abnormally low carbon dioxide production. All the individuals in the low group except No. 14 were used for a number of trials; 14 died after 105 days of starvation when only one trial had been made. Nos. 2 and 8 are females; 6 and 14 males. Females usually run lower than males (see below) but this explains only two of the cases. The detailed record of No. 6 shows a low initial period, a fluctuating intermediate period, and a very high final one as starvation progressed. The final period was characterized by a rapid loss of weight.

In the other species studied the same inverse ratio between body weight and metabolism is evident. In *Amphiuma* the result is strikingly higher for the smallest animal, and unusually constant for the three larger specimens. In the frogs the same trend may be observed, but it is not as much in evidence, probably because of the preponderance of other complicating factors.

These observations suggest that weight is not a thoroughly satisfactory basis for computing basal metabolism in these animals. The ideal basis on which to make such computations would be mass of respiring tissue in the body. In Amphibia this mass would be less in proportion to the total body weight in *Salientia* than in *Caudata*, due to the greater mass of bone in the former. This proportion would also be smaller in large animals than in small individuals of the same species, because of the increased ossification and connective tissue in the former. In man it has been found that the body surface is the more reliable criterion, and elaborate formulæ have been worked out for computing this surface from the weight and height. In Amphibia such formulæ would be useless, on account of the great variation in shape which is found between the various species. It appears that the most hopeful method of eliminating weight variations in comparing different species as to carbon dioxide output is to choose animals from each species which have approximately equal weights. In the final section of this paper an attempt is made to do this.

2. Starvation is a factor leading to important variations in the results, especially with *Necturus*. The rate of carbon dioxide elimination increases as starvation progresses. This fact has already been indicated by the observations on Necturi with

abnormally high carbon dioxide output. Table VI. and Chart 3 illustrate this variation. The two specimens chosen were both starved for a long period, and determinations were made throughout the period.

Both these animals show unmistakably the upward trend of the production of carbon dioxide with increased length of starvation, even beyond that which can be accounted for on the basis of decreasing weight during starvation. The writer was led to make the computations on the basis of standard weight because a preliminary examination of the results had suggested that the actual increase observed was largely or entirely due to this factor. These results mean, then, that the reduced amount of living tissue resulting from starvation actually produces a greater absolute amount of carbon dioxide than the greater amount of living tissue present at the beginning of starvation.

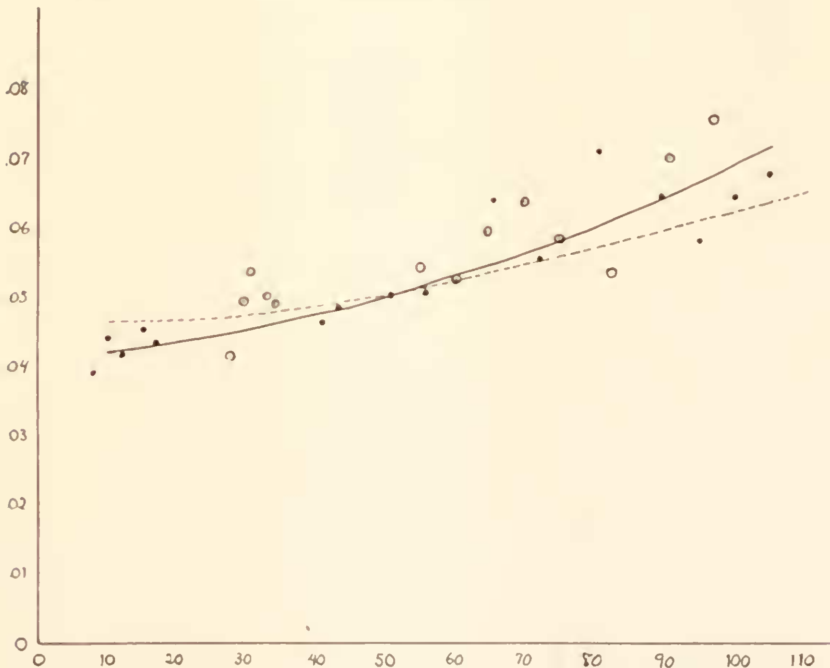

CHART 3. Influence of starvation on carbon dioxide output in *Necturus*. Abscissa, number of days starved; ordinate, CO_2 in milligrams per gram of body weight per hour. Dots refer to *Necturus* No. 4; circles to *Necturus* No. 7. The broken curve is based on the "standard weight" figures in Table VI.

Table VI.

Effect of Starvation on Carbon Dioxide Output of *Necturus*.

Necturus 4.				*Necturus* 7.			
Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.	Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.
8	139	0.0381	0.0420	27	140	0.0417	0.0463
10	139	0.0439	0.0484	29	136	0.0496	0.0535
12	139	0.0422	0.0465	31	136	0.0539	0.0582
15	138	0.0451	0.0498	33	136	0.0502	0.0542
17	138	0.0435	0.0480	34	136	0.0485	0.0522
41	129	0.0466	0.0477	55	127	0.0546	0.0550
46	127	0 0485	0.0489	60	127	0.0527	0.0531
51	127	0.0509	0.0513	64	121	0.0596	0.0572
56	123	0.0511	0.0499	70	121	0.0636	0.0611
66	121	0.0640	0.0615	75	121	0.0586	0.0562
72	120	0.0559	0.0532	82	115	0.0536	0.0489
81	118	0.0713	0.0668	91	113	0.0702	0.0630
89	117	0.0647	0.0601	97	109	0.0756	0.0652
95	117	0.0579	0.0538				
100	113	0.0646	0.0579				
104	112	0.0678	0.0603				

Results on other species are less conclusive regarding the effect of starvation. Extensive determinations were made on *Rana pipiens* with this idea in mind. No changes were noted which could be directly attributed to starvation, except that the extreme inanition previously noted in No. 2 was accompanied by a decided drop in carbon dioxide output at the end. No *Necturi* were carried as far as this. In *Rana palustris* the animals used when near death from starvation showed a much lower carbon dioxide output value than normal starving individuals (about 0.1660 as compared to 0.2730). In *Rana clamitans* the starved individual gave a lower result than the one less starved. With *Amphiuma* No. 2, the carbon dioxide elimination decreased with the weight. It seems, then, that in all species studied except *Necturus* starvation resulted in decreased carbon dioxide output per unit weight, but evidence is offered to show that the opposite is true of this species.

3. Reference to Table VI. will show that there are rather large daily fluctuations in the carbon dioxide output of *Necturus*. Daily records for individuals of other species show the same sort of variation. The possibility is not eliminated that these fluctua-

tions are the result of differençes in the aëration rate. This factor could not be accurately controlled by the apparatus used. However careful observation and comparison of the results of over 250 aërations has convinced the writer that the variations noted are not the result of differences in aëration rate. Many results were discarded in which there was good reason to believe that an abnormally slow aëration had produced a low result. Daily temperature records of the room in which the experiments were carried on were made, and these records show no fluctuations which could possibly account for the daily variations found in the results for individual animals. It is tentatively concluded that we are dealing with unexplained variations in the metabolism of the animal.

4. Sex is responsible for some minor variations in the results. It is known that basal metabolism proceeds at a higher rate in men than in women. The same appears also to be true of Amphibia. If comparable weights be chosen, it is found that *Necturi* female No. 4 is lower than male No. 7, and that female No. 8 is lower than male No. 9. *Rana palustris* females Nos. 2 and 4 are lower than male No. 3.

5. Disease was responsible for some aberrant results in *Rana pipiens*. Several of the animals (Nos. 1, 3, 7 and 8) died of a disease which the writer has called "lymph œdema." It is accompanied by an accumulation of lymph, or water, in the subcutaneous lymph sinuses, leading to a marked increase in weight and a swollen appearance. In the later stages this was invariably accompanied by capillary bursting in the skin and muscles. On autopsy the liver was spotted and the spleen enlarged and crowded with blood. All the animals that died from such œdema showed a sharp rise in carbon dioxide production at the onset of the symptoms. This production remained high until death in spite of the increased weight which would tend to reduce the carbon dioxide per gram weight. For instance No. 3 had a rise from 0.1521 to 0.2427 at the onset of the disease, and died with a production of 0.1798 mg. per gm. of weight in spite of a 25 per cent. increase in weight. No. 6 died from a complication of causes, which included a brain tumor connected with the posterior choroid plexus on the right side, a fatty degeneration which involved the entire right kidney and part of the left,

fatty degeneration of the spleen, lungs and body cavity filled with water, partial paralysis shown by inability to draw up rear legs or to support the body with the right fore leg, and a twisting of the head to the right. This animal showed a fall in carbon dioxide production from 0.1880 down to 0.1239 mg. per gm. per hour, and retained approximately the latter rate until death, in spite of a progressive increase in the severity of the symptoms. Other species did not yield much opportunity to study the effects of disease on metabolism. *Necturus* No. 12 died of fish mould. The determination made on this animal while normal yielded 0.0915 mg. per gm. per hour, while a determination made during the active progress of the infection yielded 0.1354 mg.

6. Motility of the experimental animal may be an important cause of variation in the results obtained. All the animals used were given an hour to get accustomed to the jar before each determination, and there was very little movement in the majority of cases. Salientians would shift their position occasionally, but did very little struggling. A few individuals struggled considerably during the first test made on them. The results of such experiments were discarded. It is interesting that the struggling resulted in approximately doubling the basal rate of carbon dioxide production. *Caudata* struggled very little or not at all.

A few trials were made using curare, which paralyzes the muscles, to see whether more constant results could not be obtained. It was found that the carbon dioxide production of *Rana pipiens* is thus reduced about 25 to 35 per cent., but the daily variations persist. The method described by Lund (1919) of placing the respiring animal in the jar with the barium hydroxide (suspended from the stopper in a basket) was tried on curarized animals. The results checked fairly well with those obtained on the same animals by the aëration method, but it was found that a considerable error is introduced by the necessity of removing the animal from the jar at the end of a measured time; a procedure which stirs up the air in the jar and causes loss of carbon dioxide.

VI. General Comparative Results.

A. Comparison of Classes of Vertebrata.

Table VII. has been prepared to show the cell size variations between the various classes of vertebrates, as indicated by measurements made on corpuscles from a selected representative of each class.

Table VII.

Red Blood Cell Size in Selected Species of Vertebrata.

Class.	Species.	Red Blood Corpuscles.		
		Length.	Width.	Area.
Amphibia	*Rana pipiens*	20.494	13.153	422
Reptilia	*Crotalus adamanteus*	19.0	11.0	349
Pisces	*Ambloplites ruprestris*	12.37	8.13	158
Aves	*Gallus domesticus*	12.3	6.7	130
Mammalia	*Homo sapiens*	7.9 [1]	7.9	98

The frog is below the average of red blood cell size for Amphibia, and the rock bass is below the average for fish. Man is above the average for mammals. The chicken and rattlesnake are near the average for their respective classes. The arrangement of the classes in order of increasing activity would result in the same order as that in Table VII. The avian corpuscle is nucleated, while that of the human is not. There is less difference in bulk of hemoglobin between the two than the measurements would indicate.

B. Comparison of Different Species of Amphibia.

In Table VIII. the species used in this study are listed in order of their red blood cell size, and columns are filled in for weight and carbon dioxide production. All the figures are averages of the detailed results recorded in Table I.

A study of Table VIII. shows that the general trend is clearly in the direction of increasing carbon dioxide output with decreasing cell size, but the results are complicated by the fact that the species with large cells are also large in size. To eliminate the wegiht factor, representatives of several species have been chosen which have comparable weights, and the results from these

[1] Taken from Gulliver (1875).

Table VIII.

Averages of Red Blood Cell Measurements and Carbon Dioxide Output by Amphibia.

Species.	Weight, Grams.	CO_2 Mg. per Gm. per Hr.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means	1,013.6	0.0390	61.79	36.696	3,561
Necturus maculosus	101.6	0.0814	53.416	28.617	2,401
Cryptobranchus allegheniensis			41.12	23.16	1,496
Diemyctylus viridescens			29.64	17.76	827
Rana catesbiana	511	0.07001	25.66	14.184	572
Rana clamitans	14	0.1576	22.98	11.916	429
Rana pipiens	38	0.1632	20.494	13.153	422
Rana palustris	3.3	0.1820	20.379	12.683	406
Hyla pickeringii			19.44	11.2	342
Acris gryllus			17.76	10.88	304
Chorophilus nigritus			17.96	10.04	283
Bufo americanus	6.4	0.1950	16.004	9.684	244

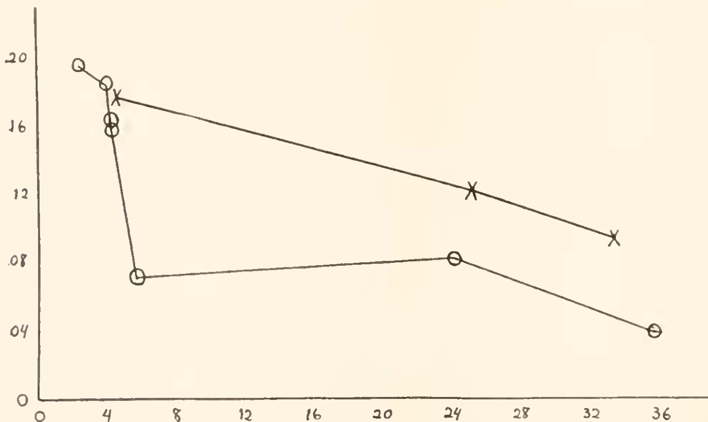

Chart 4. Cell size and carbon dioxide output in Amphibia. Abscissa, area of red blood cells in hundreds of square micra; ordinate, CO_2 in milligrams per gram of weight per hour. Curve based on circles represents the averages taken from Table VIII.; curve bases on *X* represents the individuals recorded in Table IX.

individuals have been recorded separately in Table IX. On Chart 4 are plotted two curves, one based on Table VIII., showing the general upward trend of carbon dioxide output with decreasing cell size, but obviously complicated by the weight factor; the other based on Table IX., showing the even curve which is obtained when weight variations are eliminated.

Table IX.

Red Blood Cell Size and Carbon Dioxide Production of Individuals with Comparable Weight.

Species and No.	Weight, Grams.	CO_2, Mg.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means, 2	41.4	0.0920	60.9	33.8	3,329
Necturus, 16	44.3	0.1208	50.38	31.66	2,505
Rana pipiens, 3	45.8	0.1768	21.08	14.19	470

The results recorded in Tables VIII. and IX. and in Chart 4 furnish conclusive evidence that there is a correlation between the degree of activity of a species as determined by its carbon dioxide output and the size of its red blood cells. The physiological necessity for such a correlation lies in the necessity for having sufficient surface to allow for the exchanges which take place between surface and interior. If the exchange is rapid, the surface must be large, and this enlargement of surface is brought about by having the mass divided into smaller packets.

VII. Summary and Conclusions.

1. Cell size has been measured in a number of species of Amphibia by measuring the dimensions and computing the area of red blood corpuscles. Metabolic activity was measured by collecting in barium hydroxide the carbon dioxide produced by the animal, and computing the carbon dioxide in milligrams per gram of body weight per hour.

2. Comparison of the measurements of red blood corpuscles with measurements published by other authors indicates that the measurements obtained by the present writer are too low. The cause of these low results is unknown.

3. Controls on the method used in determining carbon dioxide output indicate that this method was not extremely accurate, but sufficiently so to allow for making comparisons between the animals used. The chief sources of error were loss of carbon dioxide due to incomplete absorption, and inability to maintain a constant rate of air flow. Indirect evidence that the method is approximately correct is obtained by comparing actual results.

4. There is shown to be a size variation between the corpuscles

of the same individual, and a variation in the average size from different individuals of the same species.

5. A number of factors tend to complicate the results on carbon dioxide production. The most important is body weight, the smaller animals producing more carbon dioxide per unit of weight than the larger animals. This factor becomes especially important in making comparisons of different species, because species vary quite widely in their average weight.

Starvation in *Necturus* seems to cause an increase in the carbon dioxide output per unit of weight, even when all results on an individual are reduced to a constant weight value. In the other species studied starvation seems to lead to a decrease in carbon dioxide production.

Other factors complicating carbon dioxide output results are daily variations in the metabolism of the individual; sex, the male producing slightly more than the female; disease, usually resulting in an increased production, a fact which suggests the fever response in man; and the movement of the animal. This last factor may become of extreme importance if the animal is active, but an attempt has been made to eliminate such results by keeping the animals quiet and discarding results of determinations made on actively moving animals.

6. The conclusion that cell size varies inversely with metabolic activity is justified by the evidence presented. This is shown in a general way by comparing classes of Vertebrata, and more specifically by detailed results on twelve species of Amphibia.

The writer wishes to express his gratitude to Dr. M. F. Guyer, under whose direction the work was undertaken, and to Dr. A. S. Pearse and Dr. H. C. Bradley for their many helpful suggestions and criticisms.

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BIOLOGICAL BULLETIN

RESPIRATORY DIFFERENCES ALONG THE AXIS OF THE SPONGE *GRANTIA*.

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Differences in the rate of respiratory metabolism along the principal axis have now been demonstrated for a number of animals: for the hydroid Corymorpha (Child, '23, Hyman, '23*a*), for the medusa Cassiopaea (McClendon, '17), for Planaria (Hyman, '23*b*), and for several annelids (Hyman and Galigher, '21). Recently Shearer ('24) has reported similar results for the chick embryo and the earthworm. Unfortunately in Shearer's work regions of very different morphological constitution were compared and it is therefore doubtful if his results can be regarded as lending support to the physiological gradient conception. For example anterior and posterior halves of the chick embryo in the stages studied by Shearer differ enormously in their content of nervous tissue, due to the presence of the brain and chief sense organs in the anterior half. The much greater respiratory rate of the anterior half reported by Shearer is probably largely due to the greater proportion of nervous tissue which it contains. Similarly in the earthworm the mature head is morphologically and functionally different from the rest of the body and respiratory differences between it and other regions must be in part due to such specific differences. In brief, Shearer's measurements concern not the gradient itself but the secondary differentiations associated with the gradient. Shearer also seems to be unaware of the existence in annelids (and in the early embryos of vertebrates) of the double type of gradient (Hyman and Galigher, '17) and the pieces of the earthworm which he compared were consequently not correctly chosen and serve neither to prove nor disprove the existence in this animal

of the type of gradient which we have described for it. The acetone powder experiments do not, in my opinion, answer these objections. If the substance in such powders which absorbs oxygen is essential to respiration then it is necessarily true that types of tissues which have a high respiratory rate must contain a higher percentage of the substance. It has been repeatedly emphasized that direct proof of axial respiratory differences can be obtained only through the comparison of pieces of like morphological constitution and through the elimination of various functional factors which affect metabolic rate. It is to be hoped that Shearer will repeat these experiments with more suitable material. Such material is necessarily limited to the lower invertebrates or to the very early embryonic stages of higher forms.

The sponge *Grantia* seemed to me to constitute very favorable material for a further test of the reality of axial metabolic differences. It has the same morphological constitution throughout except at osculum and base and is more or less definitely polarized. The work was performed at the Marine Biological Laboratory at Woods Hole during the summer of 1924.

1. Method of Determining the Oxygen Consumption.—For two or three years I have been trying to devise an apparatus suitable for the study of the oxygen consumption of small organisms by Winkler's method. The method finally adopted owes its origin to a device described by Osterhaut and Haas ('17). They first suggested an apparatus separable into two pieces, one part to contain the organisms and the other part for analysis. Their apparatus is, however, clumsy to manipulate and for large animals or large amounts of material the method used by me for many years of siphoning off the sample is very much simpler and entirely satisfactory, as proved by checks. The device proposed by Osterhaut and Haas to prevent exposure to air in adding the reagents is in my opinion wholly unnecessary unless one is dealing with water of very low oxygen content. In working with small organisms, a very much smaller apparatus is required. This naturally reduces the size of the sample of water available for analysis. This difficulty is overcome by using a smaller quantity of the reagents and a more dilute thiosulphate solution for the final titration, as also suggested by Lund ('22).

The apparatus which I have been using has the following simple construction, illustrated in the accompanying figure. It consists of a Pyrex test tube *A*, without rim, 100 mm. long by 10–12 mm. diameter, capacity about 10 cc. This is surmounted by a piece of rubber tubing *B*, into which fits a short length (about 40 mm.) of Pyrex tubing *C* of the same diameter as the tube *A*. Over the end of *C* is another piece of rubber tubing *D*. The rubber tubing must fit tightly over the glass and if necessary *B* can be wired to *A* and *D* to *C*, but not *C* to *B*. A number of such outfits should be prepared. For the water blanks only the parts *A* and *B* are necessary.

Fig. 1.

The apparatus is used as follows. In those tubes which are to contain the animals all four pieces must be fitted together as in the figure and *C* must be shoved close to but not in contact with *A*. All of the tubes to be used in one experiment, including the tubes for the blanks, are filled by siphon with the same water from an elevated receptacle. The siphon should reach to the bottom of the tube and the water be allowed to flow out of the top for some time. The animals to be used can be placed in the tubes before they are filled with water if of suitable nature, or if not, can be put in after the filling. All tubes are then closed by screw clamps around *D* in the experimental tubes, or *B* in the blanks. The tubes are then placed in a suitable water bath kept at constant temperature. Those containing the animals should be agitated at intervals, in the case of non-motile animals, to prevent an accumulation of metabolic products and exhaustion of the oxygen around them. When it is desired to conclude the experiment, each experimental tube is inverted several times to insure uniform distribution of its oxygen content and is then finally inverted, the animals being brought by gravity into the section *C* plus *D*. A screw clamp of suitable size is then rapidly placed around the tubing *B* over the small interval left between *A* and *C*. On screwing the clamp, *A* and *C* will be found to move apart readily and the walls of the tubing *B* are at the same time drawn together by the negative pressure thus

developed. One should never try to pull the sections *A* and *C* forcibly apart. After screwing the clamp tightly, the sections *C* plus *D* are withdrawn from *B*, the apparatus being held inverted during the whole of the procedure to prevent the spilling of the contents of *C* plus *D*. The contents are immediately poured into a small graduated tube and the volume noted. Graduated centrifuge tubes have been found very convenient for this purpose. A little practice with the apparatus is required to prevent the spilling of the water from *C* plus *D* and a tube should be at hand to receive this water just as *C* is withdrawn from *B*. The portion *A* plus *B* is now analyzed by Winkler's method and the blanks which consist of only *A* plus *B* are analyzed at the same time. The clamp around *B* in each tube is open and 0.1 cc. of each of the reagents used in Winkler's method is added. The clamp is then closed, the contents shaken, the precipitate allowed to settle as usual, and after again opening the clamp, 0.1 to 0.2 cc. concentrated HCl added carefully so as not to disturb the precipitate. The clamp being again closed, the tube is shaken to dissolve the precipitate and the contents are then poured into a small evaporating dish and titrated with sodium thiosulphate. The latter should be about 1/500 normal. With greater dilution of the thiosulphate the end point becomes uncertain. The volume of the tube *A* plus *B* must then be determined. If care is taken not to move the clamp around *B* during the preceding operations, the tube *A* plus *B* can be filled with water of the same temperature as that obtaining during the experiment, the clamp closed, excess water at the top removed, and the contents then poured into a graduated tube. In my experience the volume of *A* plus *B* and *C* plus *D* must be determined at each experiment as they vary slightly owing to variations in the position of the clamp when the two portions are separated. One must remember to subtract 0.2 cc. from the volume of *A* plus *B*, to compensate for the loss of this amount of water when the reagents are added.

In calculating the results, it must be borne in mind that only the portions *A* plus *B* are analyzed while the animal respired from *A* plus *B* plus *C* plus *D*. The thiosulphate equivalent of each cc. of *A* plus *B* is calculated and from this the thiosulphate equivalent of the entire apparatus is obtained. It is thus neces-

sary to know the exact volume of *A* plus *B* and *C* plus *D*. The oxygen content at the beginning is determined in the same way by calculation from the water blanks and is brought by ratio to the same volume as the experimental tube. A simple subtraction then gives the oxygen consumed by the animals in terms of thiosulphate. To find the oxygen equivalent of the thiosulphate the latter must of course be standardized. A number of methods are given in any text on quantitative analysis. If the oxygen consumption in terms of volume is desired, as is usually the case, then it is desirable to conduct all of the experiments at the same temperature and to determine the volume of the tubes for this temperature. The oxygen equivalent of the thiosulphate for any given temperature can be determined from data on the volume of oxygen at different temperatures given in handbooks of physical constants.

The apparatus can be modified in various ways to suit the type of animal employed. For Protozoa the tube is closed, after filling with water and adding the animals, not by means of a clamp but by means of a glass plug. This is inserted deeply into *D* and must be firmly wired in. At the end of the experiment the tube is placed in the centrifuge in the inverted position, the bottom of *A* pointing towards the axis of the centrifuge. By centrifuging, the Protozoa are driven into the section *C* plus *D*, which is then removed as already described. For larger animals the size of the apparatus can be increased or in place of the test tube *A* a small flask of desired content can be substituted. In such cases, the amount of the reagents used and the dilution of the thiosulphate should be adjusted to the size of the sample obtained for analysis. The apparatus is not suitable for animals which cling firmly, such as planarians.

The method is naturally not as accurate as the regular Winkler method. By the analysis of duplicate samples I have found that the oxygen content of 15 cc. of water—about 0.07 to 0.08 cc. at air saturation and room temperature—can be determined with an error of about 0.002 to 0.003 cc. This makes an error of some 3 to 4 per cent. while in the regular Winkler procedure, with samples of at least 100 cc., the error is less than 0.5 per cent. The method is thus chiefly of value for comparative work.

2. Method of Determination of Carbon Dioxide Production.—For this purpose the simple method first suggested by Haas ('16) was employed. A set of standard tubes containing phenol red solutions covering the range of hydrogen ion concentration from pH 6.8 to 8.4 must be at hand. These are now sold by dealers in chemical supplies. For the experiments tubes of the same dimensions are necessary. The animals to be tested are placed in such tubes and those to be compared must be of the same weight. Phenol red in powder form is added to sea-water until the density of color is the same as that in the tubes of the standard set. An equal volume of this water is then added to each of the tubes containing the animals and the tubes then sealed with paraffin. If the inside of the tube above the water is wiped dry, paraffin will adhere to it firmly and the melted paraffin can be then added directly onto the surface of the water. The sealed tubes are placed in a water bath at constant temperature and the changes in tint due to the production of carbon dioxide by the organisms are recorded in terms of pH by comparison with the standard tubes. There is of course some error (probably about .2 pH) in working with sea-water unless the standard sets have been especially prepared for such work. This error is of no consequence in comparative work.

3. Material and General Procedure.—Only freshly collected sponges were employed and these were used as soon as brought in by the collectors. Only the cleanest and most perfect specimens were used; those selected were placed in a dish of clean sea-water and repeatedly squirted about with a pipette to free them as far as possible from foreign materials clinging to their surfaces. Unfortunately in the case of sponges it is not possible to determine by inspection whether the specimens are in good physiological condition or not. The selected specimens were placed on a glass plate, osculum and base cut off and discarded, and the body then cut into two nearly equal halves. These pieces were then placed in the tubes for the respiration tests.

It was of course necessary to weigh the pieces. They were gently rolled about on hard filter paper until they no longer wet the paper, then transferred to small weighing bottles, previously weighed, and weighed to the fourth place. In the case of the oxygen consumption tests, the pieces were weighed after the conclusion of the experiments. For the carbon dioxide pro-

duction tests, it is necessary to weigh the pieces in advance since by this method only pieces of equal weight can be compared. The sponges were cut into slightly unequal portions, and the smaller portion weighed first. The larger portion was then weighed and small pieces removed from it until its weight equalled that of the other piece. After such handling it was thought advisable to allow the pieces to stand in water for two or three hours before beginning the tests and this was always done.

The sea-water used in all of the experiments was kindly furnished to me by Dr. A. J. Goldfarb. This water was collected from the end of the Bureau of Fisheries pier at Woods Hole and stood for several days prior to its utilization to allow debris to settle. This water contained no organisms visible to the eye but no doubt some bacteria were present. As the blanks however are allowed to stand as long as the experimental tubes before analysis, this possible source of oxygen loss is cancelled out. The water was thoroughly aërated before use and was thus saturated with air at the beginning of every experiment.

In nearly all experiments two pieces of sponge were placed in each tube, such pieces being of course from the same level of the sponges concerned. Thus for each experiment two sponges were selected and cut and the two apical halves placed in one tube, the two basal halves in the other. The tubes were then filled by siphon as already described. In some cases they were filled first and the pieces of sponges dropped in afterwards. In the experiments on carbon dioxide production, the pieces were placed in the tubes and with a pipette a definite amount of sea-water colored with phenol red run into each tube. At first five cc. of water were added to each tube but the carbon dioxide production was found to be so slow that later only two cc. were employed.

The control of temperature was difficult at Woods Hole. Owing to the lack of constant temperature apparatus, the experiments had to be run at room temperature. At the beginning of each oxygen consumption experiment, the water and water bath were allowed to come to room temperature and thereafter the bath was kept at this temperature by adding warm or cold water as the case might be. The carbon dioxide experiments,

however, ran over such long periods of time in many cases, that the temperature could not be kept constant by personal attention and varied during the night with changes in the air temperature.

4. *Results*.—The results of the oxygen consumption experiments are given in Table I. Thirteen experiments were per-

TABLE I.

OXYGEN CONSUMPTION OF APICAL AND BASAL HALVES OF THE BODY OF THE SPONGE *Grantia*.

Duration of each experiment three hours. Temperature in different experiments 21 to 23° C. Oxygen given in cubic centimeters.

No. of Exp.	Kind of Piece.	O_2 Content at Start.	O_2 Content at End.	Oxygen Consumed.	Weight.	O_2 Consumed per Gram per Hr.
1	Apical	0.080	0.072	0.008	0.0193	0.138
	Basal	0.082	0.071	0.011	0.0265	0.138
2	Apical	0.077	0.061	0.016	0.0345	0.154
	Basal	0.074	0.057	0.017	0.0360	0.157
3	Apical	0.077	0.063	0.014	0.0285	0.163
	Basal	0.069	0.059	0.010	0.0319	0.104
4	Apical	0.074	0.053	0.021	0.0578	0.121
	Basal	0.074	0.049	0.025	0.0730	0.114
5	Apical	0.082	0.070	0.012	0.0682	0.058
	Basal	0.077	0.067	0.010	0.0732	0.043
6	Apical	0.080	0.069	0.011	0.0452	0.081
	Basal	0.074	0.062	0.012	0.0536	0.074
7	Apical	0.077	0.069	0.008	0.0310	0.086
	Basal	0.077	0.069	0.008	0.0482	0.056
8	Apical	0.074	0.052	0.022	0.0442	0.166
	Basal	0.075	0.055	0.020	0.0452	0.147
9	Apical	0.074	0.055	0.019	0.0388	0.163
	Basal	0.071	0.060	0.011	0.0428	0.086
11	Apical	0.074	0.053	0.021	0.0510	0.137
	Basal	0.075	0.055	0.020	0.0581	0.114
12	Apical	0.073	0.057	0.016	0.0320	0.166
	Basal	0.066	0.053	0.013	0.0406	0.106
13	Apical	0.072	0.054	0.018	0.0392	0.153
	Basal	0.073	0.051	0.022	0.0558	0.131

formed, of which one (No. 10) was lost. The table shows the oxygen content of the whole tube ($A + B + C + D$) at the

beginning and at the end of each experiment. The former is determined by calculation from the control blanks, and the latter by calculation from the analysis of the portions *A* plus *B* of the experimental tubes, the contents of *A* plus *B* and *C* plus *D* being known. When calculated in this form, the difference

TABLE II.

RELATIVE CARBON DIOXIDE PRODUCTION OF APICAL AND BASAL PIECES OF THE BODY OF THE SPONGE *Grantia*, IN TERMS OF TIME REQUIRED TO CHANGE PHENOL RED FROM pH 8.0 TO 7.5.

h., hours; m., minutes.

No. of Exp.	Kind of Piece.	Weight. Grams.	Time for Change from 8.0 to 7.5.	Remarks.
1	Apical Basal	0.051 0.058	5 h., 50 m. 9 h., 40 m.	5 cc. sea-water used.
2	Apical Basal	0.0212 0.0234	21 h., 30 m. 25 h., plus	5 cc. End point of basal piece not exactly determined.
3	Apical Basal	0.0314 0.0318	20 h., 40 m. 24 h., 55 m.	5 cc.
4	Apical Basal	0.0217 0.0214	21 h. 16 h.	4 cc. Repeated with same result.
5	Apical Basal	0.019 0.019	26 h., 15 m. 26 h., 15 m.	5 cc. Almost no difference between them at any time.
6	Apical Basal	0.0182 0.0184	15 h., 40 m. 17 h., 30 m.	4 cc.
7	Apical Basal	0.0179 0.0174	5 h., 15 m. 8 h.	2 cc.
8	Apical Basal	0.0212 0.0226	6 h., 25 m. 8 h.	2 cc.
9	Apical Basal	0.0313 0.0300	5 h., 30 m. 9 h.	2 cc. Basal piece in advance on next day.
10	Apical Basal	0.0218 0.0234	8 h., 10 m. 9 h., 40 m.	2 cc. Even next day.
11	Apical Basal	0.0112 0.0124	17 h. 19 h.	2 cc. No difference between them until after 4 hrs.

between the two values gives the oxygen consumed by the pieces. This divided by the weight and then by three (as each experiment ran for three hours) gives the oxygen consumed per gram of weight per hour. The figure thus obtained seems a little low as compared with the respiration rate of other animals

—it is about half of that of Planaria—but it must be remembered that a considerable portion of the weight of the sponge is due to the lifeless spicules.

Of the twelve experiments presented in Table I., the rate of oxygen consumption is markedly greater in the apical piece in five cases (Nos. 3, 5, 7, 9, and 12), slightly greater in five cases (Nos. 4, 6, 8, 11, and 13) and about equal to that of the basal piece in two cases (Nos. 1 and 2). In experiments 4 and 6 the difference between the apical and basal pieces is so slight as probably to be of no significance.

The experiments on carbon dioxide production yield about the same result. In nine cases, the apical pieces produce carbon dioxide at a faster rate than do the basal pieces, in one case (No. 5) the rate is equal and in one case (No. 4) the result is the reverse. The advantage in favor of the apical piece is not very great in Nos. 10 and 11.

These findings are in agreement with the electrical differences previously discovered in this sponge (Hyman and Bellamy, '22). It was found that in the majority of individuals the oscular end is electropositive (internally) to more proximal regions, but that in some individuals this potential difference is absent or may even be reversed. That these electrical differences are correlated with the metabolic differences is a view which I have held for a number of years. It is presumable that those individuals in which electrical or metabolic gradients are lacking are in poor physiological condition.

Finally attention may be called to the relation between rate of oxygen consumption and size (weight). In general, the greater the weight of the pieces, the lower is the rate of oxygen consumption. This inverse relation between size and respiratory rate seems to be general throughout the animal kingdom (cf. Hyman, '19).

5. *Summary.*—In the majority of cases, apical pieces of the body of the sponge *Grantia* consume oxygen and produce carbon dioxide at a higher rate per unit weight than do basal pieces. This result furnishes further evidence in favor of the axial gradient conception.

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THE DISTRIBUTION OF CERTAIN INSECTS OF REVERSED BEHAVIOR.*

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The recent researches of Loeb, Holmes and other students of animal behavior as well as the work of various entomologists on the reactions of insects to definite environmental factors, has given us a mechanistic interpretation of insect activities very different from the anthropomorphic interpretation of the earlier students. Shelford, Dean and others have related many insect adjustments in behavior with remarkable exactness to specific conditions of light, temperature, humidity, etc.

These reactions are frequently quite specific but many of them are the same for all the species of a genus. As, for instance, all species in a genus are nocturnal or all are aquatic. Such a generic tropism usually defines the generic habitat in a broad way. Within this general habitat the individual species will have individual habitats limited by other tropisms. Apparently the generic tropism that defines the generic habitat is seldom modified for any individual species enough that such a species may exist outside the generic habitat. But apparently a complete reversal of a generic tropism is more likely of occurrence than any lesser modification. When such occurs in a genus the individual species possessing this reversed generic tropism has entrance into an environment closed to all other members of its genus.

The writer has come across two species of insects, one a dragonfly, the other a mayfly, in which a reversal of one or more of the tropisms normal to the other species of the same genus has permitted the entrance of these reversed species into environments not open to the normal members of the genus. These finds have opened up so many interesting problems in behavior and distribution that they are well worth presenting in some detail.

* Contribution from the Department of Zoölogy and Entomology, Ohio State University, number 79.

The first of these that was found is *Æshna nevadensis* Walker of the Sierra upland of central California.[1] This is a large, pond and shallow lake dragonfly that lives at elevations of 5,000 to 9,000 feet. It is known only from the southern Sierra Mountains. It has been recorded from Walker Lake, Mono Co., California, at 7,700 feet elevation, Hardin Lake, Tuolumne Co., at 7,775 feet elevation and from Elizabeth Lake, Yosemite National Park at the great altitude of 9,000 feet. The writer found it at its optimum development in shallow, weed-filled lakes on the divide between Lake Tahoe and the Rubicon River (Calif.) where at an elevation of 7,000 feet it dominated the subalpine dragonfly life. Here in an open, meadow-like mountain pass, fairly level for about two miles, lie four shallow lakes, two flowing into Lake Tahoe and the desert drainage, while the other two empty into the Rubicon River and the Pacific drainage. On both sides rise granite crags for a thousand feet above the lakes, their lower parts green with firs, while their gray upper slopes are blotched white with fields of snow. Three of the lakes are covered with yellow flowered pond lilies and are fringed with green sedges among clumps of silver gray willows. These shallow lakes are warm because of the black peat bottom and long hours of sunshine. They support great numbers of a few species of insects—such as are able to withstand the long winters and cold nights at this elevation, as this is a subalpine situation with nightly temperatures at or near freezing. Eight other species of dragonflies are common here. These are northern forms that occur in the mountains of Oregon at 3,000–4,000 feet elevation and in British Columbia at sea level.

The habitat of *nevadensis* appears to be entirely above that of all other species of North American Æshnas. It appears as strayed individuals at 4,500 feet but does not appear in numbers until an elevation of 6,000–7,000 feet is reached. From here up to 9,000 feet it appears at its optimum development. No other Æshnas have been found regularly at these higher altitudes. From about 4,500 feet down to sea level, however, Æshnas are a constant element of the North American Odonate fauna. Twenty or more lowland species have been described from north of Mexico while there are actually seven or eight species living

[1] Kennedy, *Proc. U. S. Nat. Mus.*, Vol. 52, pp. 483–635.

about the lower slopes of the Sierras. Thus *nevadensis* has a habitat entirely above or outside of that of the other North American members of the genus.

We do not know the behavior complex of *Æshna nevadensis* in enough detail to speculate on all the adjustments in reactions necessary to adapt this species of a lowland genus to a highland habitat. It shares with all other Æshninæ, and without any change, a positive reaction to shining water surfaces when the sexual instinct is not overbalanced by hunger. This is a reaction through the eye as *Anax*, a related genus, was found reacting just as positively to the glistening surfaces of the crude oil pools of the Bakersfield (Calif.) oil field where hundreds of *Anax junius* perished while mating and trying to oviposit in the crude oil. When hunger predominates over the sexual impulse the reactions change so that the Æshnas fly away from the water on hunting trips into the surrounding territory. They fly away from the water and roost on trees when the minimum flying temperature is reached, also at twilight on warm evenings when the minimum flying light is reached. The minimum flying light varies greatly with the different species as some will still fly when it is so dark to the human eye that the dragonfly can be seen only when it is outlined against the sky or some white surface.

As ponds for oviposition are the same on the Sierra upland as at sea level, we find the reactions of *nevadensis* while under the sexual impulse practically identical with the similar reactions of the lowland species. The adjustments to the upland come in the reactions of the insects when hunger and other impulses outweigh the sexual impulses, and have nothing to do with the coursing of the males and females over the surface of the water while mating and ovipositing.

Two of these adjustments to the conditions found in these high altitudes are quite obvious. First the hunting individuals react negatively to the warm stratum of air next the ground so that, except early in the morning when the ground stratum of air is still cool, they hunt high off of the ground flying from fifteen to one hundred and fifty feet in the air. It is a tree-top species. This positive reaction to cool air probably explains the attraction of *nevadensis* to this alpine habitat. All other species of Æshna,

when hunting, react negatively to the cooler situations of the habitat, the warmer the place the better, until 100–105° is reached when some species begin to become inactive.

The second obvious adjustment necessary is in the time of emergence. All lowland species of Æshna, as far as known, emerge at night, those the writer has reared, *umbrosa* and *constricta*, emerging at or near midnight. This appears to be an adjustment of value in that the imagoes have hardened sufficiently by daylight for flight and so escape the blackbirds and other marsh fowl that enjoy soft freshly emerged insects. But at an elevation of 7,000–9,000 feet with snow fields spread about, the nightly temperatures are never far above freezing and frequently below, making night emergence precarious as the young dragonfly would be too chilled to crawl out of its nymphal skin. Here a second adjustment to this elevated habitat appears. The emerging nymph of *nevadensis* instead of being negatively geotropic in the dark is negatively geotropic in the light, so that the winged adult emerges in the broad daylight when the temperature is high enough to insure a successful withdrawal from the nymphal skin. The writer found numerous individuals emerging in the bright sunshine in the early afternoon hours.

Thus we see that *Æshna nevadensis* has left the general warm lowland environment of the genus and has entered an entirely different region through having two of the tropisms normal to Æshna reversed.

While in the mountains of eastern Tennessee last spring, the writer discovered a mayfly, *Ephemera guttulata* Pictet, which appears to occupy a habitat entirely different from that of any other species of Ephemera. It too appears to have certain of its reactions the reverse of those of the other species of this genus.

Ephemera guttulata is a most interesting mayfly in several ways. It is one of our large mayflies. Its wings are so heavily clouded that at a little distance they appear almost black, especially as contrasted against the abdomen, which is immaculate snow-white. This bizarre insect lives in the smaller of the perennial, spring-fed mountain torrents that flow down the higher of the Eastern Tennessee Mountains. On Chilhowee Mountain these streams pour down deep V-shaped ravines over beds of small stones and coarse grit, in a succession of miniature waterfalls, for they

descend at a rate of several hundred feet to the mile. These mountains are covered with pines on their high, dry ridges but the deep ravines between these ribs of pine woods are filled with a dense growth of deciduous timber so that the torrents are heavily shaded by tall trees in their whole course.

The burrowing larva of *guttulata* lives in the meager areas of coarse sand and muck found in the little basins below the waterfalls. The subimago emerges during the day. Those the writer observed came out on dull cloudy days. These fly out of the shade over the stream, through the surrounding brush and up to the better lighted areas of the hill side where they rest in the full light. The dull gray subimago then sheds a thin skin and comes out a fully developed imago with its brilliant black and white colors and its sexual maturity. No observations were made as to whether this occurred on the day of emergence or the following day. Because of the few subimagoes seen it probably occurred the same day as the emergence. Unfortunately also, no mating dances were seen. These probably occurred among the tree tops, in the deep dusk, just before egg laying began.

When the evening twilight had deepened to the point where it became difficult to pick one's way along the streams, the females of *guttulata* would appear over the little pools hurrying back and forth about a foot above the surface of the water apparently laying eggs. Their conduct was more like that of female dragonflies than like the usual hurried visit of the mayfly female dropping all of her eggs in a single effort. No males were caught at these times over the streams.

It was in these flights in the dense twilight gloom of the bottoms of these mountain gorges that the probable value of the bizarre coloration came to mind. The enormous development of the eyes, the evidence of the rudimentary antennæ together with certain experimental work indicate that the major reactions of the mayflies are through the sense of sight. Except for the white abdomen, the mayflies, at the time of these twilight flights, were practically invisible to the observer. These white abdomens, as the *guttulata* females doged about in the gathering darkness, reminded one of the streaks of light of a flight of fireflies. Apparently then this white abdomen is useful to *guttulata*

in the mating flights in the deep shade of the mountain gorges as its visibility is very obviously increased by this color pattern. A snuff-colored, lowland Ephemera would be practically invisible under the same conditions. It is interesting to note that of several species of mayflies flying on these streams at this time *guttulata* is the latest on the wing in the evening and flies after the others have ceased. Some of the smaller species fly a full hour earlier, so that, though, of dull colors they are quite visible.

We can check this series of reactions of *guttulata* by a comparison with the reactions of the other species of Ephemera, all of which inhabit either large open streams or lakes. Probably the reactions of *Ephemera simulans*, the common Lake Erie species, are best known. The nymph of this species burrows in the mud of the lake bottom, being obviously negatively phototropic. At the time of emergence it becomes positively phototropic and rises to the light of the sky. At Put-in-Bay this emergence takes place between eight and ten P.M. It sheds its skin as it rises through the ten to thirty feet of water so that on arrival at the surface it bursts out fully winged, when it becomes less positively phototropic and flies toward the dark land. It rests on the shore vegetation until the following evening when it sheds its final skin, becomes sexually mature and at twilight flutters up and down in the mating dance. At this stage it is evidently becoming positively phototropic again. In this twilight nuptial dance it leaves the dark foliage for the more open lighter spaces. The males grasp the females and release them after a contact of a few seconds. The female becomes at once completely, positively phototropic and flies out toward the light surface of the lake to deposit her eggs.

If we compare this series of reactions with those of *guttulata* of the shaded mountain streams, we find that two of the series of reactions of the latter are reversed. *Guttulata* is negatively phototropic as a nymph, is positive as it emerges, but *remains positively phototropic after emergence* as it flies from the heavily shaded creek to the lighter areas above the shade. Further, *after copulation it becomes negatively phototropic* and flies down to the densely shaded torrent to oviposit. Any of the open stream species of Ephemera would react themselves away from the

shaded stream when they started to oviposit. So by these reversed reactions *guttulata* is able to occupy a habitat that the normal members of the genus *Ephemera* are not able to occupy, one that is ecologically outside of the general habitat of the genus.

Certain experimental studies in the behavior of mayfly nymphs point to possible explanations of some of this series of reactions. Wodsedalek [2] has demonstrated that the nymph of Heptagenia is negatively phototropic. Also that CO_2 in the water will make a nymph which has been negatively phototropic, reverse its reaction and become positively phototropic. It is then possible that the change in reaction at the time of emergence, when the nymph ceases to burrow and swims up to the light, is due to an accumulation of CO_2 in the nymph after its tracheal system has detached itself from the gills in the last nymphal ecdysis. The shedding of the skin actually starts by a general loosening of the chitin several hours before the final emergence occurs. During this time, if the gills are early detached, much CO_2 could accumulate in the tissues, enough eventually to reverse the phototropism and so cause the nymph to rise to the surface.

On emergence the nymph fills its tracheæ with air and simultaneously becomes negatively phototropic, so that it flies toward dark land. It appears to retain this reaction until the next evening at dusk or for about twenty hours when it mates and the female at once becomes positively phototropic and flies towards the light surface of the open lake. During the intense nuptial dance her tissues have been accumulating CO_2 and in some way the sexual orgasm overbalances the condition, giving the acid condition full sway.

The experiments of Allee and Stein [3] show that the reactions are not as simply explained as they have been sketched in the preceding paragraphs. The actual intensity of the light probably also figures in some of the reactions. Krecker's work [4] in the subimagoes of *Hexagenia*, a close relative, shows that these, in spite of flying to the dark land, are positive to certain bright

[2] Wodsedalek, "Phototactic Reactions and their Reversal in the Mayfly Nymphs of *Heptagenia interpunctata* (Say)," BIOL. BULL., Vol. 21, pp. 265–271, 1911.

[3] Allee and Stein, "Light Reactions and Metabolism in Mayfly Nymphs," *Jour. Exp. Zoöl.*, Vol. 26, pp. 423–458, 1918.

[4] Krecker, "Phenomena of Orientation Exhibited by Ephemeridæ," BIOL. BULL., Vol. 29, pp. 381–388, 1915.

lamps. Anyone who has seen the snow storms of mayflies that come to the street lamps of the lake ports realizes that the subimago can have a reverse tropism under such conditions.

We are beginning to recognize physiological species among insects—those based on habits and habitats. In parasitic insects particularly we recognize generic reactions to common hosts so that we unofficially recognize physiological genera. There is no reason why we should not, except the expediency of morphological characters. Viewed in this light *Æshna nevadensis* and *Ephemera guttulata* are physiologically outside of their respective genera.

When the writer first thought through the habits and distribution of the North American species of the genus *Æshna*, his conclusion was that the positive thermotropism of the lowland species was perhaps different in degree and distinct for each species, thus explaining the restriction of each species to its specific thermal belt. Thus it appeared, at first, that this difference in positive thermotropism accounted for the fact that some Æshnas lived in hot Arizona, others in the cooler northern states, while still others are restricted to the northern parts of Canada and Alaska. However on investigating further this does not appear to be true.

The restriction of each species to its specific thermal belt is probably not due to a limitation of the positive thermotropism of the adult to the narrow limits of the particular thermal belt inhabited by that species. This is quite contrary to Merriam's theory of distribution by thermal zones.[5]

This distribution of the various species in different thermal zones however is a very striking thing and some of the thermal conditions are easily sketched. Except for *mutata*, *californica* and *multicolor*, which are early spring species, the majority of the species of Æshna are on the wing in August, so the writer has worked out the flying temperatures for August at four points, Yuma, Arizona, St. Louis, Missouri, St. Paul, Minnesota, and Sitka, Alaska. Each of these is representative of the flying conditions for a restricted group of Æshnas. The Yuma temperatures apply to *jalapensis*, *multicolor* and *arida*. The St. Louis

[5] Merriam, "Life and Crop Zones of the United States," Bull. U. S. Biol. Survey, No. 10, pp. 1–79, 1898.

temperatures apply to *constricta*, which is the only species broadly distributed across the central states, while the St. Paul temperatures apply to a series of several species such as *interna*, *canadensis*, *umbrosa*, etc. Under the Sitka temperatures we find *sitkensis*, *subarctica* and *septentrionalis*. Such species as *palmata* and *constricta* are found in two or three of these zones.

To define the flying temperatures the mean maximum and mean minimum day temperatures for August have been taken from the Weather Bureau Report for the above stations.[6] Ten degrees was then added to the mean maximum day temperature to give the approximate maximum sun temperature, which is the temperature to which the local dragonflies react positively, while the mean minimum day temperature remained unchanged, as it is a shade temperature to which the local dragonflies react negatively. Thus these temperatures show roughly the range of day temperatures which the species of *Æshna* meet during their flight season in each of the general zones represented by the temperature records. These records for August are as follows:

	Mean Maximum in the Sun.	Mean Minimum in the Shade.
Yuma, Arizona	114°	77°
St. Louis, Missouri	96°	69°
St. Paul, Minnesota	90°	60°
Sitka, Alaska	72°	45°

From the above it is obvious that the temperatures during flight do differ greatly for the various species of *Æshna*. However our observations indicate that all lowland Æshnas are always positive to heat so that the higher the temperature, the greater the activity of the insect. Hine's observations[7] on Kadiak Island were that *palmata* was most active on the warmest days. Walker's observations[8] on the Canadian species are that increased temperatures always increased the speed of Æshnas but his observations do not include temperatures above 90°. Somewhere above 90° there may be a limit for this increase of

[6] "Climatology of the United States," Bull. 2, 1906. Temperatures for Yuma, St. Louis and St. Paul. *Monthly Weather Review*, Dec., 1898, p. 549. Temperature for Sitka.

[7] In conversation with the writer.

[8] Walker, p. 33. "The North American Dragonflies of the Genus *Æshna*," Univ. of Toronto, Biol. Studies, Biol. Series, No. 11, 1912.

speed as the temperature rises, which limit may be actually reached by some of the southern species on very warm days. The writer [9] has made one interesting observation in this regard. *California* and *multicolor* on very hot days when the temperature is from 100° to 105° take frequent rests, by hanging up in the shade every few minutes. This was noted in the Yakima desert at Sunnyside, Washington. Apparently, for these at least, somewhere in the higher nineties a temperature is reached above which increase is depressing and no longer stimulating. However we can safely say that for all lowland species of *Æshna* an increase of temperature up to 95° increases activity so that all are equally positively thermotropic.

The thermal distribution of the four groups of species outlined above must then be conditioned through some indirect check on the life history. Some temperature condition of the water for the nymph or the developing egg may be the limiting factor rather than the flying temperature for the imago. Undoubtedly individuals of each species continually fly beyond the limits of the optimum habitat but the offspring of such do not survive or we would have a spreading species. This constant pressure of the species of dragonflies into surrounding but unsuitable habitats was worked out by the writer [10] on Put-in-Bay Odonata in 1922. Until we know the life histories of the various species in minute detail we will not be able to define all of these limiting factors except as we stumble onto them accidentally.

A further conclusion appears indicated from this study. As all Æshnas, except *nevadensis*, have one type of reaction to temperature in the imago, and all the Ephemeras, except *guttulata*, have one type of reaction to light and the reactions of these two odd species are just the reverse of the other species in their respective genera, we may conclude that a given type of insect nervous system can be completely reversed from its usual reaction more easily than it can be modified in a lesser degree. This would appear logical from the theoretical grounds of the mechanics of the nervous system. Because of its minute size, the insect nervous system is characterized by the relatively

[9] Walker, see ref. 8, p. 33.

[10] Kennedy, "The Ecological Relationships of the Dragonflies of the Bass Islands of Lake Erie," *Ecology*, Vol. III., pp. 325–336, 1922.

small number of units (either cells or combinations of cells) that comprise it. Such simple nervous systems obviously cannot produce the extended series of finely graduated reactions that are possible to a more complicated type of system. On the other hand the mechanism is already present for the reversal of any particular reaction. So a complete reversal of a generic reaction which puts the species entirely outside of the normal generic habitat, is more likely than the slight modification necessary to put it into a near but only slightly different habitat in which it would be held by only a slight gradation of the general reaction.

At first thought, if it is easier to have a reversed reaction in the insect nervous system than to have a graduated reaction, one might think that the genus would fly apart as to any unity of environment, so that each species would have a habitat strikingly different from that of each other species, that there would be no such thing as a generic habitat. Observation shows that this is not so. Species are superimposed and habitats overlap in a most confusing manner and in any large genus there is usually an easily recognized generic type of habitat. So far not enough experimental work on behavior has been carried out to determine if the species of any one genus of insects are distributed by graduated reactions to one type of stimulus. However an analysis of some of the factors of the problem appear to indicate that such a distribution, for instance as the species of *Æshna*, show in a series of temperature gradations, may not be due to a slight difference in reaction to a specific stimulus, but may be merely an apparent series each species of which is held in its particular zone by some different positive or negative reaction to any one of a variety of stimuli.

An insect with incomplete metamorphosis passes through two stages, nymph and adult, in each of which it may pass through several physiological stages of development. The nymph has at least two, its feeding stage and its quiescent preëmergence stage. The imago has at least five stages, the teneral, the sexually mature stage, which can be divided into periods of hunger, periods of sexual lust, periods of egg-laying, and finally the stage of senility. The last need not be considered in a problem of species distribution as it is beyond and outside the genetic cycle. In each of these stages several stimuli control the individual,

the reaction to anyone of which may be the factor that limits the species to its particular environment. If we consider only ten of the more easily recognized tropisms then in the six physiological stages enumerated above we have one hundred and twenty positive and negative possibilities for a reaction to an individual stimulus that might limit the species to a specific habitat. The problem is really much more complicated than this as many more reactions enter and all are more or less conditioned by morphological factors. So there is no difficulty at all in accounting for a great variety of factors, any one of which may limit the species to a rather narrow habitat.

In building the conception in the student's mind of the group of habitats occupied by all the species of a genus the mind automatically picks the most obvious habitat characteristic that is common to all the habitats under consideration. If this habitat characteristic, such as flying temperature for *Æshnas*, varies from habitat to habitat the mind automatically considers each species delicately adjusted to the exact degree of this variation in its specific habitat. This may not be true at all. And the factor which limits each species may not be the obvious one but some inconspicuous factor and it may be in each species in the genus a factor from a different category. It may be a temperature factor in one, a moisture factor in another, a chemical factor in a third species, etc. Thus graduated generic factors may largely be a compound figment of the mind of the observer.

THE INDIVIDUALITY OF THE GERM-NUCLEI DURING THE CLEAVAGE OF THE FERTILIZED EGG OF THE ROTIFER, *ASPLANCHNA INTERMEDIA*.[1]

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DAVID D. WHITNEY.

Recently while studying sections of the rotifer, *Asplanchna intermedia* (Hudson and Goss), double nuclei were discovered in the fertilized eggs while only one nucleus was seen in the parthenogenetic eggs. All of the cells in the early cleavage stages showed the two nuclei but in the later stages fewer and fewer cells contained two nuclei. It was decided to study the sections in an attempt to determine how late in the embryonic development the two nuclei remain distinct.

This species produces small parthenogenetic eggs developing into males, larger parthenogenetic eggs developing into females and large fertilized eggs which also develop into females. Both kinds of parthenogenetic eggs may be distinguished readily by their very thin covering membrane. It appears to be no thicker than the covering membrane of the blastomeres of an embryo. (Fig. 1, *A–G*). The covering of the fertilized egg is many times thicker and also shows a different structure than that of the parthenogenetic eggs. By noting the covering membranes alone one can distinguish these two kinds of eggs at a glance. (Fig. 1, *A*, *D*, *H*). No observer has ever found a parthenogenetic egg with a thick shell in this species.

When the male parthenogenetic egg is fertilized it becomes larger, develops this thicker characteristic shell and is popularly known as the winter egg. The origin of both of these eggs is identical but their mature appearance depends upon whether they are fertilized or not. They both produce two polar bodies while the female parthenogenetic egg only produces one polar body.

[1] Studies from the Zoological Laboratory, The University of Nebraska, No. 142.

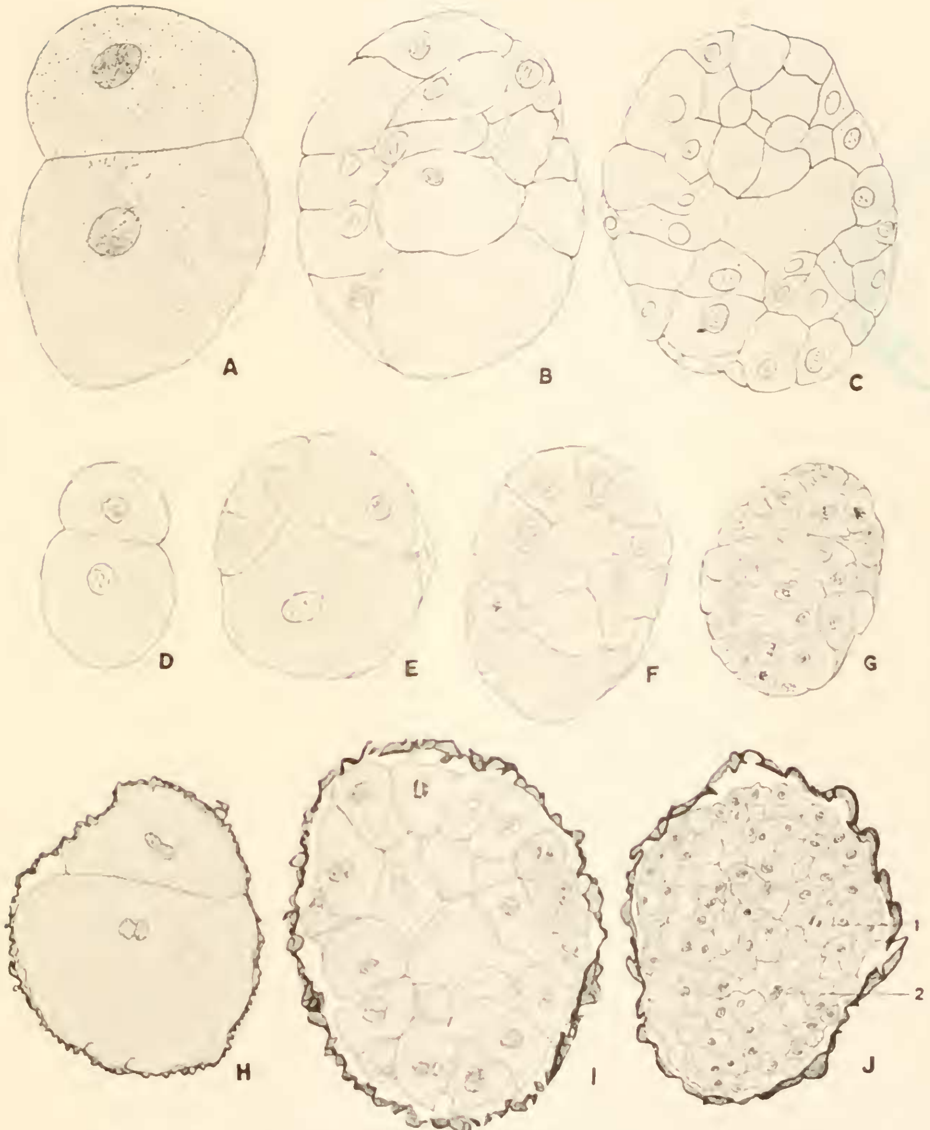

FIG. 1. *A–C*, sections of early embryos developing from parthenogenetic female-producing eggs showing one nucleus in the cells; *D–G*, sections of early embryos developing from parthenogenetic male-producing eggs showing one nucleus in the cells; *H–J*, sections from embryos developing from fertilized eggs showing two nuclei in some of the cells.

As this form of rotifer is oviviviparous and the females frequently carry from 1–10 developing embryos and young it was found practical to fix entire individuals in the killing fluids. Flemming's strong solution for about one hour was used. Then the material was bleached, dehydrated and imbedded in clove oil and paraffin. Large numbers of individuals were sectioned in masses, cutting sections 5 microns in thickness and the favorable sections studied. These animals were raised in great numbers in laboratory cultures in weak horse-manure solutions. The figures were outlined by means of a camera lucida with a magnification of about 430 diameters and the details filled in free hand.

As it is the male parthenogenetic egg that can be fertilized and changed into a larger and thicker shelled egg it is of some interest to compare the nuclei of the cells in the embryos developing from these eggs without fertilization and with fertilization. For a complete comparison the female parthenogenetic egg has been included.

Each cell of the embryo developing from both kinds of the parthenogenetic eggs contains only one nucleus regardless of whether it is in the 2-cell stage or in later stages of a larger number of cells (Fig. 1, *A–G*). Many hundreds of sectioned embryos have been observed in the various celled stages and not one has been found whose cells contained more than one nucleus.

A few somatic cells were found in which the chromosomes could be counted fairly accurately in the embryos developing from both kinds of the parthenogenetic eggs. The small male embryos developing from the male parthenogenetic egg showed the haploid number of about 25 and the larger female embryo developing from the female parthenogenetic egg showed the diploid number of about 51 chromosomes (Fig. 2, *A–B*). Although the chromosomes are very small and somewhat crowded together their exact number is reasonably certain. Tauson working on this same species has concluded the haploid number in the mature male parthenogenetic egg to be 24 and the diploid number of 48 in the female parthenogenetic egg.

In the early embryos developing from the fertilized eggs two distinct nuclei appear in each cell. These nuclei usually appear apposed to each other but clearly distinct (Fig. 1, *H*). In later stages only some of the cells show the two nuclei and as the

embryo becomes older and is comprised of many more cells very few of the cells are found containing two nuclei, as for instance about the 250-cell stage only two such cells were found in a section (Fig. 1, *I–J*). Beyond that stage no cells have been found containing two nuclei.

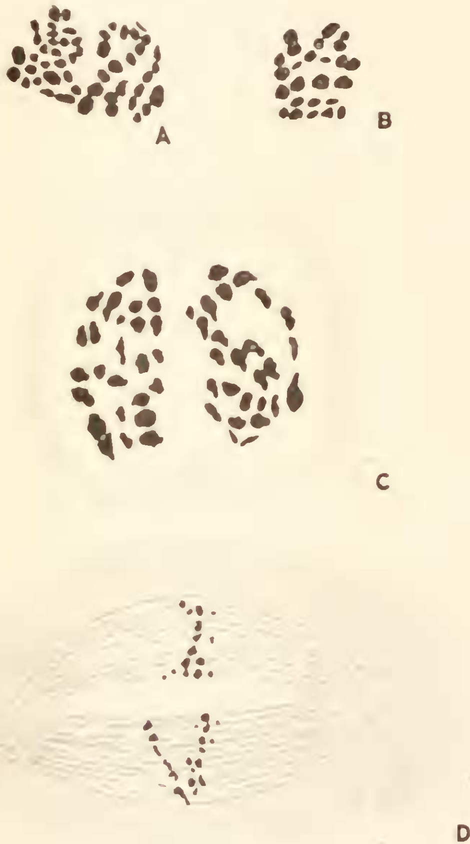

FIG. 2. *A*, somatic cell of a female embryo developing from a parthenogenetic egg showing 51 chromosomes; *B*, somatic cell of a male developing from a parthenogenetic egg showing 25 chromosomes; *C*, the two nuclei of a matured and fertilized egg showing 26 chromosomes in each nucleus; *D*, a double spindle from a somatic cell of an embryo developing from a fertilized egg. Drawings somewhat diagrammatic because of minute size of chromosomes.

Mitotic figures occur showing double spindles which seem to indicate that the two nuclei divided independently of each other in cell division (Fig. 2, *D*). Why this occurs only in the cells in

the early stages of the embryo is not determined. Perhaps as the cells become more numerous and consequently smaller the size ratio between nucleus and cytoplasm is so altered that there is only room for one mitotic figure and consequently both nuclei are crowded into one fused nucleus.

A section was found of a fertilized egg in the one-cell stage in which the chromosomes could be counted fairly accurately. Each nucleus seemed to have 26 chromosomes. This fact clearly differentiates it from either of the parthenogenetic eggs. It also furnishes additional evidence to support the claim that the fertilized egg was originally the male parthenogenetic egg which has been entered by the sperm. The haploid number of 26 chromosomes is found in the mature male parthenogenetic egg and in each of the two nuclei of the fertilized egg. One of these nuclei in the fertilized egg is undoubtedly the egg nucleus and the other is from the sperm (Fig. 2, *C*).

Further observations of the sections of the fertilized eggs show that in the first cleavage each nucleus gives rise to two groups of chromosomes each of which passes separately to the daughter nuclei (Fig. 2, *D*). During the ensuing resting stage each germ nucleus is represented by a structurally distinct vesicle. Thus the separateness of the germ nuclei is maintained throughout the entire nuclear cycle. In mitosis there seem to be two spindles each with its distinct set of chromosomes which separate regardless of each other. Probably each group of chromosomes splits into halves, and a maternal and a paternal group go to each end of the double spindle, so that each daughter cell receives two sets of chromosomes around which separate walls are formed, and a maternal and a paternal vesicular nucleus appear.

The double nuclei have been found up as far as about the 250-cell stage. In one section of an embryo there were 103 visible cells and among these two had double nuclei (Fig. 1, *J* 1–2).

Two distinct nuclei in fertilized eggs and their developing embryos have been observed in other forms by various other workers. Mark observed this phenomenon in *Limax*, Van Beneden in *Ascaris*, Häcker in *Cyclops*, Conklin in *Crepidula*, Beard in *Raja batis*, Smith in *Cryptobranchus allegheniensis* and other workers have seen indications of it in other forms. No

one has observed this in rotifers previously nor have the two nuclei been seen in as late stages as in this species of rotifer.

Summary: It has been found in *Asplanchna intermedia* that the germ nuclei in the fertilized egg do not fuse in the early cleavage stages, but each gives rise to two sets of about 26 chromosomes, one set of which pass into each of the daughter nuclei. Each nucleus is a structurally distinct vesicle.

In later cleavage stages the nuclei become fused so that in about the 250-cell embryo only a very few show the two nuclei in one cell.

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THE RELATION OF CARBON DIOXIDE TO SPONTANEOUS MOVEMENTS IN THE LARVÆ OF *OPSANUS TAU*.

HENRY C. TRACY.[1]

Spontaneous movements in embryos have been reported in the literature since the time of William Harvey (1651). Such movements have been observed in many groups of animals, and their occurrence is probably a universal phenomenon.[2]

More or less definite statements as to a relation between embryonic movements and respiratory conditions, have appeared several times in the literature.

Balfour ('76) says: "The band [of longitudinal striated muscles] developed at this stage [*H*] appears to be a special formation which has arisen through the action of natural selection, to enable the embryo to meet its respiratory requirements, by continually moving about, and so subjecting its body to fresh oxydizing influences; and as such affords an interesting example of an important structure acquired during and for embryonic life."

Ahlfeld ('05) showed the existence of rhythmic movements in the human fetus during the second half of pregnancy, manifested by rhythmic undulations of the maternal abdominal wall. These movements were near the rate of respiration in the newborn and were interpreted as preliminary respiratory movements.

Sarwey ('15) states that there is an increase in the strength and frequency of fœtal movements at the beginning of asphyxia and shortly before death.

Minkowski ('21) says: "On admet generalment que l'asphyxie de la mere mene a une augmentation des mouvements fœtaux, l'asphyxie directe du fœtus pourrait donc egalement le faire. Le manque d'oxygene agirait alors comme une excitation inter-

[1] Contribution from the laboratory of the United States Bureau of Fisheries, Woods Hole, Mass., and the Department of Anatomy, University of Kansas.

[2] References to the literature on these movements may be found in Preyer, '85; Munkowski, '21, and Wintrebert, '20.

ieure provenant du sang, et determinerait ou favoriserait du moins les mouvements du fœtus."

Wintrebert ('14) states that in the trout the embryonic contractions are more frequent and lively in a medium containing CO_2 than in one containing oxygen. He considers ('20, pp. 450), these movements favorable to respiration, excretion, and circulation. He also states that lack of oxygen depresses the movement (in Selachians).[1]

Experimenting with cat fetuses eight to nine centimeters long, T. Graham Brown ('14) observed that rhythmic alternating movements of the limbs (interpreted as progression movements) arose spontaneously and could also be produced by asphyxia resulting from pressure on the umbilical cord.

In adult animals, the relation between the respiratory center and CO_2 has long been known, even though the exact mechanism is still in doubt. Stimulating effects of asphyxial conditions on blood pressure and motor mechanisms through the spinal cord have been reported (Mathison, '10 and '11).

A. D. Waller ('96) found an increased irritability in nerve fibers treated with CO_2, the effect varying with different concentrations. Roaf ('12) showed that the rate of branchial movements in fish showed a positive correlation with the H-ion concentration and with the concentration of CO_2; the appendages of barnacles showed a negative correlation to these conditions.

These observations and other well known physiological studies indicate that stimulation by metabolites occurs in many different varieties of contractile and transmissive mechanisms; it, therefore, may be expected that alteration of body fluids with respect to respiratory conditions may have some relation to the endogenous movements of embryos, and in fact it is possible that changes in such conditions may be the most important factors in the production of these movements.

The experiments reported in this paper were undertaken in order to test the effect of different concentrations of CO_2 in the sea water on the spontaneous movements of larvæ of *Opsanus tau*.

The yolk sac, with the network of capillaries over its surface,

[1] So far as his descriptions go, the depression of movements which he observed appears to have taken place under asphyxial conditions and were probably due to the narcotic action of a considerable excess of CO_2 (Wintrebert, '20, pp. 324 and 328).

furnishes favorable conditions for an interchange of substances between the sea water and the blood of the larvæ and probably acts as a respiratory organ before the branchial mechanism becomes functional. It is, therefore, comparatively easy for purposes of experiment to change certain chemical characteristics of the body fluids of the larva by altering the character of the sea water around it.

Opsanus tau (toad fish) is an inactive bottom fish which inhabits shallow, sheltered water. The eggs are found in such localities, fertilized and in the process of development, attached to the under-side of sticks, stones, tin cans, etc. The objects to which the eggs are attached may be brought into the laboratory where they develop in an apparently normal manner if furnished with a constant supply of fresh sea water. The eggs hatch in 3 or 4 weeks; the yolk sac, however, remains attached; about three weeks later, the larva has absorbed nearly all the yolk; it then becomes loosened from its attachment and swims free.

In this species the first observable movement is the heart beat which begins in specimens of 12 to 14 somites. The earliest stage at which muscular movements of the body were observed was in a specimen about 19 somites. Movements appear in the first few somites (2 to 4 or 5); the somites contract slowly and apparently simultaneously, causing a lateral bending of the anterior body region.

These movements take place singly and at irregular intervals, often with several minutes between and with no apparent relation between successive movements. Responses to external stimuli, however, do not take place until a much later period, in fact, not until after hatching. There seems, therefore, no question that these early movements are the result of changes in the internal conditions.

Soon, however, there appears a slight tendency for movements to appear in groups; this tendency increases until at the time of hatching, in addition to occasional single bendings of the body, right and left alternating coils of the body often take place very rapidly for a brief interval, producing a kind of vibratory or "fluttering" movement of the whole body. These "fluttering" movements become more and more predominant during the larval stage. When the free swimming period begins the "flut-

tering" movements of the body which alternate with irregular intervals of rest have now become regular undulating vibrations which cause short bursts of swimming movements of the fish through the water.

It is evident that the spontaneous movements of the embryo are gradually elaborated into the swimming movements of the free larva. Similar bursts of motion at the end of considerable intervals of rest on the bottom are characteristic of the adult throughout its sluggish existence. In the other teleosts which I have observed (*Fundulus* and *Tautogalabus*), the embryonic movements occur with much greater frequency and with only very brief intervals between; during development these movements become merged into the continuous type of movements characteristic of fishes of pelagic habits. It would appear that the activity habits of these animals are not widely different at any stage of their existence, and are determined by some internal physiological mechanism, the earliest expression of which is found in the spontaneous movements of the embryo.

Spontaneous movements of the mandible and branchial mechanism begin soon after hatching; they are, at first, slight and irregular, but gradually, in the course of about 5 days, they develop into the respiratory rhythm. Exteroceptive and proprioceptive reaction mechanisms do not respond to external stimuli at the time of hatching but gradually become functional during the larval period.

Preliminary experiments were carried out in the summer of 1922 at the Marine Biological Laboratory at Woods Hole.[1] Newly hatched larvæ were exposed to sea water from which all the gases were removed by passing hydrogen for an hour and a half through sea water which had been previously boiled.

Of 13 specimens, under these conditions, 11 showed a decrease in spontaneous movements. In another set of experiments 8 larvæ were exposed to sea water in which a number of *Fundulus* had been allowed to remain until they showed signs of asphyxiation; of these, 6 specimens showed a decided increase in spontaneous movements. In another experiment, 6 specimens were placed in KCN *n*/1000 in sea water. In all of these specimens,

[1] The research room at the Marine Biological Laboratory was supplied me from the Graduate Research Fund of the University of Kansas.

the movements were immediately stimulated to such an extent that they were nearly continuous during the first 15 minutes; they slowed down gradually, reaching about normal at the end of the first hour; during the next following 15 minutes, the movements ceased entirely. The results of these experiments show a close resemblance to reactions which would have been expected from a respiratory mechanism under similar conditions.

During the summer of 1923 at the laboratory of the United States Bureau of Fisheries,[1] at Woods Hole, I attempted to carry out more accurate experiments by subjecting *Opsanus* larvæ to sea water of known definite partial pressures of CO_2. For suggesting this method I gratefully acknowledge my indebtedness to Doctor Homer W. Smith. Details of the method have since been published (Smith and Clowes, '24).

Methods.

The method of experiment consisted essentially in adding different percentages of HCL $n/20$ to the sea water in which the larvæ were placed. The changes in CO_2 partial pressure, and the H-ion concentration produced by this means are stated in the tables.

In applying this method, the following sources of error had to be taken into account:

(1) Variation in frequency and duration of the spontaneous movements in different individuals, and in the same individual at different times.

(2) Temperature changes.

(3) Stimulation by manipulation and handling of the embryo previous to the beginning of each experiment; previous observations had shown that manipulation of the yolk sac which results from handling of the larvæ, pressure of flowing water, etc., may cause an increased movement even though the larvæ do not respond to direct tactile stimulation.

[1] In connection with laboratory arrangements in the conduct of these experiments I am greatly indebted to Dr. R. E. Coker, Director of the Laboratories of the Woods Hole Station of the U. S. Bureau of Fisheries.

(4) Stimulation of movements which might result from the shaking the bottle containing the specimens, after the addition of the acid to the sea water.

To control these sources of error, the following routine procedure was adopted. The larvæ were taken from the stock in the aquarium, scraped off from their attachment, and placed in a 250 cc. flask of fresh sea water at 25° C., and allowed to remain undisturbed from one half hour to an hour. The bottle containing the larvæ was then shaken a definite number of times (20 uniform back-and-forth movements). This procedure served as a control for the shaking after addition of the acid; several experiments seemed to show that stirring of the sea water in this manner had no appreciable effect on the movements of the larvæ, but nevertheless, at the beginning of each record of normal movements the bottle was shaken as described. The movements of the larvæ were then recorded for a period varying from 15 minutes to one half hour; this furnished the record of the movements under normal conditions of each individual at 25° C. At the end of this time, a little sea water was poured out of the bottle, the proper amount of HCl, *n*/20 added and the contents of the bottle made up to exactly 250 cc. with sea water and the stopper inserted. These operations were carried out as rapidly as possible in order to prevent the escape of CO_2 into the air. The bottle was then stirred (as described above for the normal control) in order to secure as rapidly as possible a uniform CO_2 pressure and H-ion concentration throughout the water in the bottle. The movements were then recorded, usually for 30 minutes to one hour. At the close of this period, the acidulated sea water was poured off, and replaced with fresh sea water of the same temperature. The slight agitation of the larvæ which was unavoidable in pouring off the acidulated water is probably a sufficient control for the stirring at the beginning of the previous steps in the experiment. A record of the movements was then made.

In making the record, the time, character and extent of the movements were noted. A single coil to one side was taken as the unit. The number of separate movements can be recorded in this way with considerable accuracy; but in the case of movements following each other in rapid succession, as in the

"fluttering" movements mentioned above, a somewhat arbitrary method is necessary. These "flutters" were assigned a value of 10, which seems about the minimum estimate of their value. At the free swimming stage these "flutters" sometimes continue several seconds which, of course, cause an extended swimming movement; a value of 10 for each second was given for such movements. The results thus arrived at are partly based on estimates and hence cannot be exact. But they will serve to give the general order of magnitude of the movements of the larvæ under different conditions. Most of these movements are undoubtedly greater than the minimum assigned value; hence the error favors a negative result. Since the method of estimation of numerical value was the same for the same larva under both control and experimental conditions, errors due to the method of recording tend to neutralize each other.

The records of each experiment were afterward transferred to chart records by recording the total numerical value of the movements for each minute on cross-section paper. The records of all specimens for each concentration of CO_2 were then averaged for each minute; then, in order to smooth the curves somewhat, averages for each two minutes were taken and recorded as shown on the charts accompanying this paper.

Observations were made at two periods of the larval development:

(1) Within the first five or six days after hatching and before external respiration had begun (larvæ about 6 or 7 mm. in length). At this stage there is no response to light, rotation, jar, or vibrations and little or none to tactile stimuli.

(2) At the end of the larval period when the yolk sac is nearly absorbed, and the larvæ are on the point of swimming free (18–20 mm. in length). All the reaction mechanisms, as far as known, are now functionally developed.

The behavior of the respiratory mechanism in the free swimming larvæ (second period) was observed under the different conditions of CO_2 and the record superimposed on the charts recording the body movements. The time in seconds necessary for the completion of 10 respiratory movements was taken with the stop watch for each individual (whenever possible the

average of 3 observations for each minute), and the average of all the individuals for each concentration of CO_2 was indicated on the charts for each two minutes by the number of spaces above the base line.

Results.

In the first stage 3 or 4 specimens can be studied at the same time in one experiment; but in the free swimming stage only one specimen can be recorded at a time. The breeding period of this species is brief, hence, the number of individual records obtainable is not as large as is desirable, but when the averages are brought together the conclusions stated below seem justified. But the number is insufficient to give a smooth contour to a curve indicating the effect of any given concentration with respect to the time of exposure or to state in an exact manner the effect of different concentrations.

The chart records indicate the following relation between changes in the partial pressure of CO_2 and the body movements and respiratory rate.

A. Body movements.

1. The movements in each group show an increase during the first few minutes following an increase in the concentration of CO_2.
2. When the specimens in the water containing the higher concentration of CO_2 are returned to normal sea water, the movements in each group are depressed considerably below the normal. This depression is very pronounced during the first 10 minutes and gradually approaches the normal during the 30 minute period.
3. The intensity of the reaction varies with the increase in CO_2, up to the middle range of concentration; above this the intensity of stimulation decreases with the increase of CO_2 concentration.
4. The reaction to increased CO_2 is less intense but of greater duration in the earlier than in the later stage. On returning to normal sea water the depression of movement is less in the earlier than in the later stage and recovery is slower.

The different effects in the two stages are not easy to explain.

They might possibly be due to the presence of the large yolk sac in the younger stage. The mass of the larva at hatching is considerably less than that of the yolk; the yolk then must act as a "buffer" and the increased CO_2 is "soaked up" from the blood by the yolk substance inside the vitelline net work nearly as rapidly as the blood absorbs it from the water outside. The increase in concentration in the body fluids of the larvæ is, therefore, relatively slight at first and increases slowly until the yolk substance approaches equilibrium with the sea water relative to the CO_2 concentration. It is only in the high concentrations that this can take place during the first 10 minute period. In returning to normal sea water the blood begins to absorb the CO_2 back from the yolk and hence the depression period is pronounced.

At the beginning of the free swimming stage the amount of yolk remaining is very slight, and in the gills active interchange of gaseous substance is taking place directly between the sea water and the body fluids of the larva; the effect of increasing the concentration of CO_2 in the sea water is, therefore, almost instantaneous and hence the stimulation rapidly reaches its maximum, followed quickly by the depression due to effects of excessive CO_2. The recovery in the normal sea water is rapid on account of the rapid attainment of equilibrium between the inside and outside of the larva through the branchial system.

It is, however, difficult to understand why it should require so long a time for the yolk substances to take up the CO_2. According to the combined record of the newly hatched larvæ (Chart XII.) the heightened reaction lasts about 25 minutes, but only 10 minutes in the free swimming (Chart XIII.).

There are, of course, other differences between the two stages. In the first there is a relative preponderance of undifferentiated tissue, as compared with the later stage. The chemical relations of young tissue must be different from older. The larvæ will endure asphyxial conditions better than the adult (see p. 19). Anaërobic respiration is known in the young of other forms. Susceptibility to strychnine is less in young than in adults (Schwartze, '22). Apparently, young tissue has greater adjustment capacities to factors affecting a fundamental activity like metabolism than older tissue.

It must be stated that there are a few aberrant specimens which fail to react in the usual manner. The number and incidence of these are shown in the tables. Some of these exceptions are only apparent, since most specimens show movement either while the bottle containing them is being shaken or in the interval (a minute or less) immediately afterward, before the record can begin. These movements, of course, do not appear in the record. Also, there seem to be more aberrant specimens in the higher concentrations because of the stronger narcotic action of CO_2 and its more rapid onset. Some are perhaps pathological in spite of careful effort to select only healthy specimens; there is a large mortality under laboratory conditions, perhaps 50 per cent. during the larval period. Most of these exceptional specimens are probably thus explained; whether all can thus be accounted for, is uncertain; there may be physiological conditions in which an increased CO_2 may produce a depression immediately instead of stimulation.

B. Rate of Respiratory Movements.

In the lower ranges of concentration of CO_2, the respiratory rate is faster during the period of exposure (Charts VI., VII., VIII.). The middle ranges of CO_2 concentration (Charts IX. and X.) appear to result in a balance between the stimulating and depressant effects. In the higher ranges (Chart XI.) after a short period of stimulation, the rate falls considerably below the normal. In the extreme concentrations (addition of 3.5 per cent.–4 per cent. acid to the sea water—Chart XIV.) the respiration becomes irregular, then of the Cheyne-Stokes type; after a time it gradually improves and becomes regular, though proceding at a somewhat slower rate than in the normal. Reuss ('10) has shown the stimulating effect on the respiratory rate of moderate concentration of CO_2 in adult fish and the depressant effect of higher concentrations. There is, therefore, a tendency for these higher concentrations to break down the respiratory system; the capacity for recovery is probably dependent on some secondary reserve mechanism. This capacity for recovery seems not to be possessed by the spontaneous movements.

On return to normal sea water, the effect of the lower concentrations (Charts VI., VII.) is the same on the respiratory rate as on the movements, that is, a depression below normal;

in the case of the higher concentrations, the respiratory rate rises above the normal on return to normal sea water.

Obviously there is a close similarity between the effect of CO_2 on the rate of movements of the body and rate of respiratory movements in the lower concentrations; in the higher concentrations the respiratory mechanism has a compensatory capacity both during exposure and in recovery afterward, which is not possessed by the motility mechanism. Apparently, then, the regulation of respiration and motility at ordinary concentrations of CO_2 takes place through similar mechanisms; in high concentrations, the respiratory system can bring into play secondary reserve mechanisms, which appears to be beyond the capacity of the system controlling body movements.

The resistance of these larvæ to asphyxiating conditions is very great. One of these larvæ was left over night in a bottle containing the highest concentration of CO_2; the next day the specimen lay motionless on the bottom of the bottle with the respiratory rate about the same as shown on the preceding day; on putting the specimen into fresh sea water the respiratory rate quickly jumped above the normal, the body movements approached the normal in 12 to 15 minutes. Larvæ placed in water from asphyxiated *Fundulus* (which causes acute symptoms in half grown adult toad fish) apparently suffered no inconvenience; in boiled sea water, through which hydrogen had been bubbled for one and a half hours, the early larvæ show no effects during the first hour and a half (aside from reduction of body movements) although oxygen was entirely absent. Evidently anaërobic respiration is possible for a considerable time at least, in these embryos.

Stockard ('21, p. 173) indicated the probability that double and abnormal embryos in fishes may be produced by asphyxial conditions at certain stages. In the toad fish I have found only one double larva, although in the course of several seasons I have examined several thousand specimens. This high degree of resistance to asphyxial conditions is no doubt correlated with the crowded stagnant condition of the water in which this species passes its embryonic and larval life.

That there is some definite relation between spontaneous movements and respiratory movements is shown by the reduction

in the rate of respiratory movements which seems always to exist immediately after each spontaneous movement (Chart XV.). At the end of each movement the branchial apparatus is motionless for a few seconds; it begins activity very slowly, and gradually resumes the normal rate in 10 to 15 seconds. This phenomenon may be due to acapnia induced by the rush of water through the gills while the larva is in motion; furthermore, if it is the production of metabolites which stimulates the body movements, the same substances would probably increase the volume of blood flowing through the branchial vessels and thus favor the washing out of CO_2 from the blood; a brief inactivity of the respiratory mechanism then ensues, due to the lack of stimulation.

Speculation as to the details of the mechanism by which embryonic movements are stimulated and depressed under the various conditions described in this paper seems premature until the relation between specific effect of CO_2 and variations in the H-ion concentration has been experimentally determined. Experiments with KCN, with different concentrations of CO_2, and with CO_2 removed, indicate that essentially similar mechanisms regulate respiratory and body movements, except for some accessory reserve system which exists in the case of respiratory movements.

The relation of different concentrations of CO_2 to the amount of motility must be a significant factor in determining the migrations and habitat of fishes. Shelford, Wells and Powers, (Shelford, '23) in their work on the relation of H-ion gradients to the movement of fishes apparently did not study the mechanism of the reactions. The results which these writers report are perhaps due to the direct effects of H-ion changes in the body fluids on the primitive neuro-muscular system, or, in other words, to variations in the rate of the endogenous, "spontaneous" movements conditioned by alterations in the amount of CO_2 or H-ions in the surrounding medium. According to this view, the optimum H-ion concentration is automatically determined by the effect of different parts of the acidity gradient on the endogenous movements, and not to any "choice" or "selection" on the part of the fish.

A question of broader significance is raised by these experi-

ments since the results suggest that endogenous movements may be stimulated by variations, local as well as general, in the concentration level of metabolites which result from differences in the metabolism rate in different regions of the embryo. Jacobs ('22, p. 25) has made a similar suggestion regarding the production of pseudopods in *Amœba*.

Summary and Conclusions.

1. Newly hatched and free swimming larvæ of toad fish (*Opsanus tau*) were subjected to different concentrations of CO_2 produced by additions of different percentages of HCL $n/20$ to sea water.

2. Increased concentration of CO_2 is followed by an increase in the endogenous (spontaneous) body movements in both stages; in newly hatched larvæ the reaction of CO_2 is less intense but of greater duration (average about 25 minutes) than in free swimming larvæ (average about 10 minutes).

3. On return to normal sea water from the higher concentration of CO_2, the frequency of the body movements is depressed below the normal; the depression is less in the newly hatched larvæ than in the free swimmer and the recovery is slower.

4. In the lower ranges of CO_2 concentration, the body movements (in both stages) and the rate of respiratory movements (free swimmers) vary with the increase in CO_2; in the higher ranges the body movements, after a period of stimulation, remain depressed far below the normal, while the respiratory movements, after a brief stimulation followed by depression and irregular rythm, recover and proceed regularly at a little below the normal rate.

5. On return to normal sea water, the body movements for all concentrations of CO_2 remain depressed (about 30 minutes for the free swimmers, longer for the newly hatched larvæ); the rate of respiratory movements is below the normal for the lower concentrations, but is increased above the normal on return from higher concentrations.

6. Respiratory movements and spontaneous body movements react similarly to the lower concentrations of CO_2 and hence their regulation probably takes place through a similar mechanism; at the higher concentrations, the respiratory system

appears to bring into play a secondary reserve mechanism which gives it a compensatory capacity not possessed by the neuromuscular system through which body movements are produced.

7. Toad fish larvæ are much more resistant to asphyxial conditions than adults.

8. It is suggested that the migration of fishes in a H-ion gradient is probably conditioned by the effect of acid substances on the endogenous body movements.

9. It is suggested that stimulation by variation in the concentration level of metabolites produced inside the body may be the source of endogenous (spontaneous) movements.

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Explanation of Tables and Charts.

Charts I. to V. are records of specimens just hatched, before the beginning of respiration.

Charts VI. to XI. are records of specimens at the beginning of the free swimming stage.

Chart XII. is the combined record of the movements of the just hatched larvæ under all concentrations of CO_2.

Chart XIII. is the combined record of the movements and respiration rate of the free swimming larvae under all concentrations of CO_2.

Two minute spaces are marked off on the horizontal base line: height of the line indicates the average amount of movement (estimated as described on p. 10) made in each minute of the two-minute period by the specimens in each concentration of CO_2. The average amount of movement each minute under normal conditions for the time observed (15 minutes to 1 hour) is indicated by the arrow at the beginning of each chart, marked A. N. M. The arrow marked A. M. R. indicates the average movement per minute when the larvæ are returned from the acidulated water back to the normal.

In Charts VI.–XI. and XIII. the respiration record of the free swimmers is superimposed on the movement record (respiration not being established in the just hatched larvæ). The height of the short horizontal line in each two minute space indicates the number of seconds (usually the average of three observations in each minute) taken for 10 respiratory movements (average for all the specimens of each concentration of CO_2). Hence a drop in the line means increased respiratory rate, and vice versa. The observed normal rate (average for each minute) is indicated at the beginning of each chart by an arrow marked A. N. R.

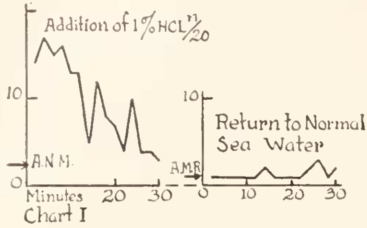
The arrow marked A. R. R. indicates the number of seconds required for 10 respiratory movements when returned to normal sea water from the acidulated water.

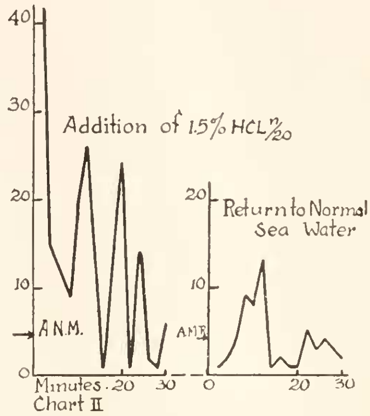
Chart XIV. shows the influence of high concentrations of CO_2 on the respiratory rate in two individuals (*A* and *B*).

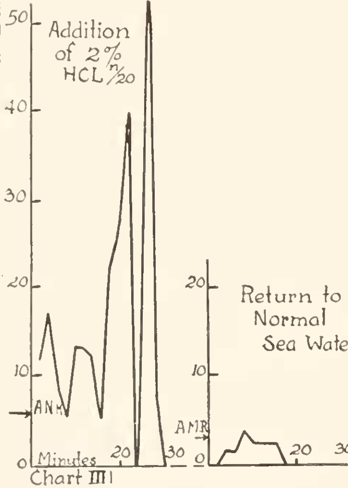
Chart XV. shows the fall of the respiratory rate which is observed at the end of each spontaneous movement.

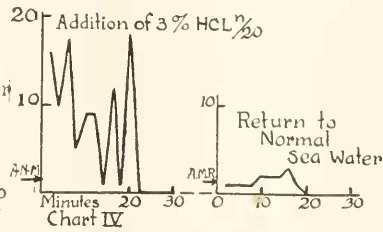
On Tables I. and II. the average number of movements (that is, the number of separate movements which took place regardless of extent and character) is given for each 10-minute period under different conditions.

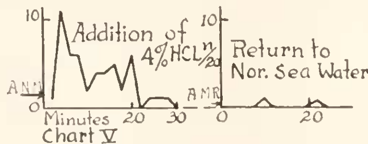
In Tables III. and IV. is given the average numerical value (that is, the amount of movement, using the coil to one side for unity) for each 10-minute period under different conditions.









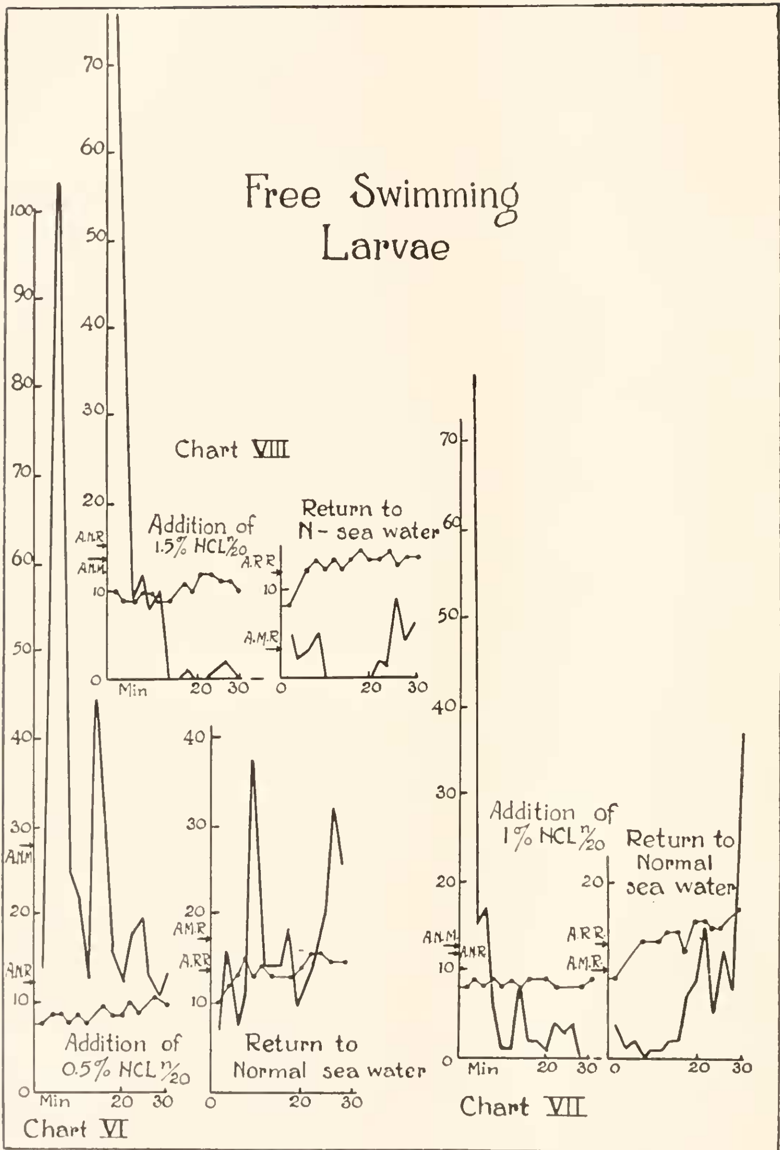


CHARTS I–V.

Table I.

Number of Movements in 10 Minute Periods of Newly Hatched Larvæ under Different Concentrations of CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% HCl n/20.	Partial Pressure CO_2.	P_H	Number of Specimens	Average Number of Normal Movements in 10 Minutes.	Average Number of Movements Each 10 Minutes under Increased CO_2.				Average Number of Movements per 10 Minutes when Returned to Normal.			
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	Number of Specimens not Stimulated.	Number of Specimens.	1st 10 Minutes.	2d 10 Minutes.	Number of Specimens Aberrant.
1	.010	7.0	13	6	9.6	4.7	5.4	4	8	1.4	3	2
1.5	.0185	6.64	4	5.15	10.3	3.5	4.5	1	4	6.3	7.7	2
2	.025	6.42	8	13.2	13.5	6.7	4	3	6	9	17.3	2
3	.038	5.96	9	11.4	11.4	7.5	0.7	4	9	10.2	7.6	2
4	.052	5.00	4	3.4	9.5	1.5	1.5	0	4	0.25	0	1
Averages and totals.			38	8.4	10.9	5.3	3.5	12	31	5.4	7.3	9

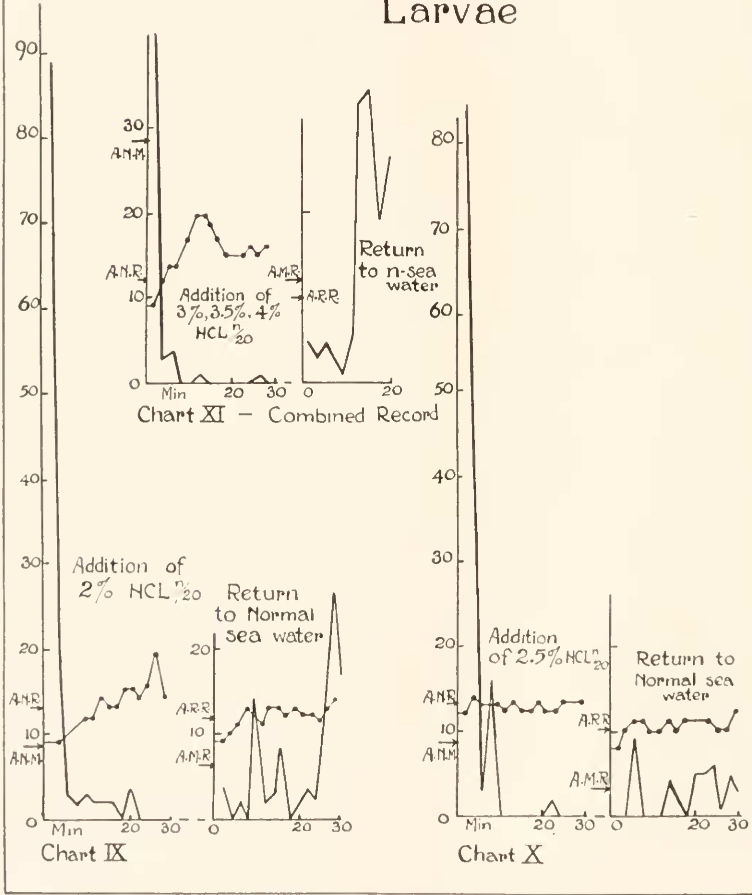


CHARTS VI–VIII.

TABLE II.

NUMBER OF MOVEMENTS IN 10 MINUTE PERIODS OF FREE SWIMMING LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% HCl n/20.	Partial Pressure CO_2.	P_H	Number of Specimens.	Average Number of Normal Movements in 10 Minutes.	Average Movements of Each 10 Minutes with Increased CO_2.			Number of Specimens not Stimulated.	Average Movements per 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.		1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
0.5	.005	7.5	3	21	46	17.7	22.3	0	15	15	26.5	0
1.0	.010	7.0	3	11.1	15.3	3.3	2.5	2	3.3	11	25.7	0
1.5	.0185	6.64	3	14.3	24.3	3.3	1.7	1	3.3	0.3	9.3	0
2.0	.025	6.42	4	14.2	8.2	4.5	1.8	2	4.0	2.5	12	0
2.5	.0315	6.2	2	10.3	10.0	0	0	1	3	3	6.5	
3.0	.038	5.96	1	20	6	0	0	1	15	22	—	0
3.5	.045	5.60	1	1.3	6	0	0	0	2	1	22	1
4.0	.052	5.00	2	27.5	2.5	0	0.5	2	25	3	10	0
Averages and totals.			19	15.4	17.2	4.6	5.2	9	8.1	6.5	16	1

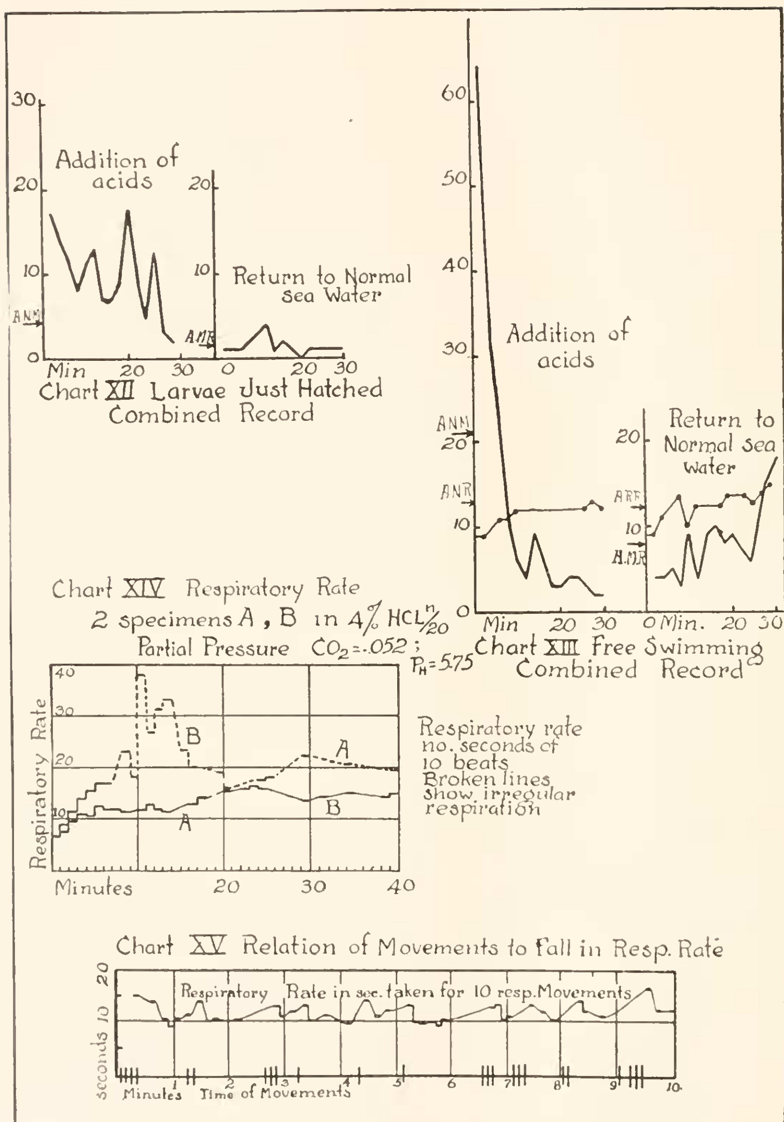


CHARTS IX–XI.

TABLE III.

NUMERICAL VALUE IN 10 MINUTE PERIODS OF NEWLY HATCHED LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
% HCl n/20.	Partial Pressure CO_2.	P_H.	Number of Specimens.	Average Number of Normal Movements in 10 Minutes.	Average Numerical Value of Each 10 Minutes under Increased CO_2.					Average Numerical Value per 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	No. Specimens not Stimulated.	Number of Specimens.	1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
1	.010	7.0	13	41.6	154.7	103.2	51.2	2	8	5.9	6.5	18.7	2
1.5	.0185	6.64	4	50.2	193	148	48.2	1	4	48.5	49.5	51.2	1
2	.025	6.42	8	68.4	112.4	53	—	4	6	22	15.1	—	0
3	.038	5.96	9	24.7	113.1	39.4	—	1	9	11.1	22.5	23.5	0
4	.052	5.00	4	24.5	42.2	42.2	6.7	1	4	0.3	0.0	0.8	1
Averages and totals.....			38	42.3	128.1	75.8	42.1	9	31	15.3	17.5	22.8	4



CHARTS XII–XV.

Table IV.

Numerical Value in 10 Minute Periods of Free Swimming Larvæ under Different Concentrations of CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% of HCl $n/20$.	Partial Pressure CO_2.	P_H.	Number of Specimens.	Average Number of Normal Movements in 10 Minutes.	Average Numerical Value of Each 10 Minutes under Increased CO_2.				Average Numerical Value Each 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	Number of Specimens not Stimulated.	1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
0.5	.005	7.5	3	287.4	497	219.6	195.3	1	162.3	130.6	156	
1.0	.010	7.0	3	247	249	21	27		21	47	168.0	1
1.5	.018	6.64	3	137.3	337	4.6	6.3	1	25.3	0.6	63.6	
2.0	.025	6.42	3	157.8	196.2	16.2	2.7	2	48	20.7	125.3	
2.5	.0315	6.2	3	93	267.3	3.3	0.3	1	20	23.3	53.5	
3.0	.038	5.96	1	245	43	0.0	0.0	1	107	18.8	0.0	
3.5	.045	5.60	1	8	60	0.0	0.0		20	20.3	0.0	1
4.0	.052	5.00	2	503	117.5	0.0	2.0	1	18	0.0	0.0	
Averages and totals.......			19	211.9	261.9	41.7	36.2	7	52.2	37.1	89.5	2

THE RELATION OF BODY TO ENVIRONMENTAL TEMPERATURES IN TURTLES, *CHRYSEMYS MARGINATA* BELLI (GRAY) AND *CHELYDRA SERPENTINA* (LINN.).

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Although a considerable number of workers have recorded their observations on the relation of body temperature to that of the environment of different so-called cold-blooded animals, only a comparatively few of these refer especially to the reptiles, as a group, or to the turtles in particular. With but the exceptions indicated below, all the records from the early workers were based upon a limited number of individuals taken for the most part at random within narrow limits of normal but slight environmental changes. It has thus become quite generally assumed by biologists that turtles together with other cold-blooded animals approximate the temperature of their surroundings. In view of the fact that turtles as a representative group of the reptiles have an unique phylogenetic position, spanning the gap as it were between the warm-blooded birds on the one hand, and the cold-blooded amphibians on the other, it was thought that a study of their body-temperature changes when followed through high and low critical temperatures as well as the ordinary non-critical ranges, might yield interesting data.

The earliest observation of the body temperature in turtles is probably recorded by Walbaum (1), in 1782. He found the temperatures differed only one or two degrees from that of its environment and fluctuated with it. Following Walbaum a considerable number of workers made similar observations during the first half of the nineteenth century. Milne-Edwards (2) in 1863, after giving a critical review of the work done on these forms by Czermach (3), Murray (4), Tiedemann (5), Davy, J. (6), and Valenciennes (7), concluded that the body temperatures

varied but little from that of the environment wherein the limits did not exceed 4 degrees C. It is of interest that the same author noted the observations of Valenciennes (7) which were later substantiated by Sclater (8), that the female python when coiled about the eggs during incubation, maintained a body temperature considerably above that of the surrounding air. Sclater found on comparing the body temperature of the incubating female with that of the male, that during the height of incubation the difference was as much as 10 degrees C., and that she was some 20 degrees C. above the surrounding air. Whether this marked increased in heat production was brought about by bringing into play a thermal regulatory mechanism, or was due to other causes was not stated and has not yet been learned. Similar observations were reported in 1881 by Forbes (9), with somewhat less conspicuous differences. The greatest difference of the air and the surface coils of the snake was about 6 degrees in the male, and about 9 degrees C., in the female. It was noted in this study that the female took no food and was comparatively inactive for weeks before and during incubation.

In 1903, Martin (10) working with the respiratory exchanges in Monotremes and Marsupials included some observations on the blue-tongue lizards (*Cyclodus gigas*). These, five in number, he carried through changes in temperatures varying from 5 to 39 degrees C., within a calorimeter. At room temperature they were comparatively active but became quite inactive at 5 degrees. On warming they increased in activity up to until about 30 degrees and above this their activity diminished. The body fluctuations accompanying these changes were noted. Throughout the middle ranges (10-35 degrees C.) the body temperature was a function of the environment but the CO_2 production was fairly constant. At the extremes (below 10 and above 35) as shown in his plots, Fig. 3, sharp breaks occur, with approximation to Van't Hoff's law. Notwithstanding that he kept the animals in an environment of between 39 and 40 degrees C., for over two hours he was unable to get their mean temperature above 38.5. In their work on certain of the cold-blooded animals Rogers and Lewis (10) followed the body temperature fluctuations in only two representatives of the vertebrates, the fishes (goldfish) and the amphibians (sala-

manders) but concluded that neither of these forms possessed mechanisms for either heat production or heat loss.

Experimental.—Turtles were collected in the vicinity of Lakeside Laboratory, Lake Okoboji, Iowa. The painted variety was unusually abundant in July and August in the shallow cove at Miller's Bay. The snappers were comparatively rare and could not be obtained in considerable numbers. Most of these were

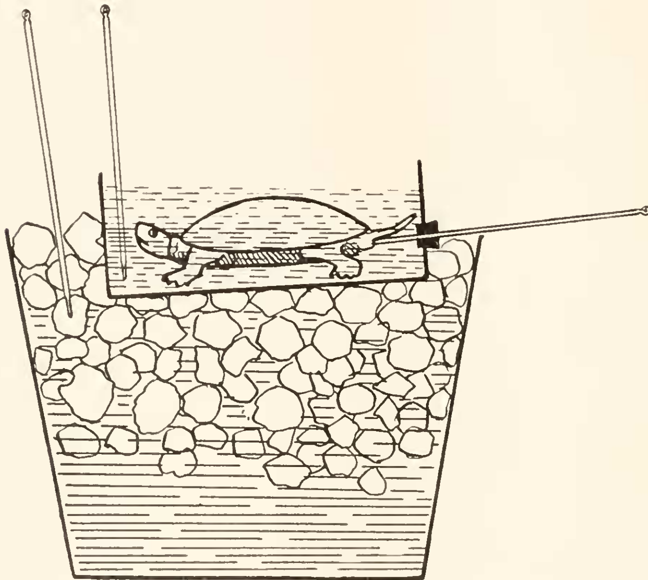

FIG. 1. Diagram of relationships of the apparatus used in cooling experiments. The animals were made fast by clamp in the smaller container surrounded by water, and this subjected to an ice bath. Temperatures were read from the three thermometers shown.

taken from Hottes' and Marble Lakes, west of Spirit Lake, while others were found along the shores of Little Sioux river and in the neighborhood of Hanging Bog. Suitable specimens of various sizes were placed in a live box as caught, and from there were taken into the laboratory as needed.

Individuals were weighed and mounted in such a way that their temperature changes might be recorded. Those placed in air as a surrounding medium were fixed to a board by heavy rubber bands slipped about the carapace and plastron and spaced so that the animal was unable to release itself by kicking.

The board was then supported at either end high enough above the table that the animal could not get sufficient traction to crawl. In this position the tail could be tied back and the thermometer inserted in the rectum.

Experiments with water as the surrounding medium were more difficult to carry out, and involved the construction of special containers which could be adapted to different sizes of individuals (Fig. 1). Shallow tin dishes of convenient sizes were found to fit the purpose, allowance being made so that a stoppered thermometer could be supported at the side. In mounting, the thermometer could be slipped into the rectum, and the animal made fast by adjustable wire clamps. Mounted in this way the specimen could be subjected to an ice mixture and its changes in body temperature as well as those of the environment be recorded.

Although some slight variations in the experimental technique were followed for checking purposes, in general the procedure in all cases involved the tabulating of the date of the experiment and the time intervals of the observations, the room, as well as the immediate environmental and the subject's temperature, the subject's number and its weight, and remarks on the behavior and stages of activities of the animal, under the conditions of the experiment. Some forty experiments exclusive of several preliminary observations, furnish data for this report, these being apportioned about equally between the two forms of turtles studied. For convenience, the discussion of results are grouped under the following captions:

Body Temperature Fluctuations in the Non-critical Ranges.— In a series of thirteen experiments, the diurnal and nocturnal fluctuations in body temperature were taken on individuals of the painted and snapping varieties, both in air and water. A typical chart of observations taken at intervals over a twelve-hour period from a painted turtle mounted in air, weighing 485 grams is given in Table I.

In general it is noted that the body temperature lags from one to three degrees as the temperature rises, and remains slightly above as it is lowered, being slightly effected by the state of the activity of the animal. The same fluctuations are apparent during the nocturnal intervals. Similar observations were made

upon the snapping turtles in air and in water but since comparable results were obtained through these ranges it is unnecessary to cite typical protocols.

TABLE I.

AUGUST 1, 1924. OBSERVATIONS SHOWING THE TYPICAL FLUCTUATIONS OF BODY TEMPERATURES WITH ENVIRONMENTAL TEMPERATURES IN THE PAINTED TURTLE.

Date.	Time.	Room Temp.		Subj. Temp.		Diff.		Activity.
Aug. 1	8.00 A.M.	63° F.	17.5° C.	61° F.	16.0° C.	−2.0° F.	−1.0° C.	Quiet
	9.00	63	17.5	61.5	16.3	1.5	−0.8	"
	9.30	63	17.5	63	17.5	0	0	Active
	10.00	64	18	64	18	0	0	"
	10.30	65	18.5	64	18	−1.0	−0.5	"
	11.00	67	19.5	65	18.5	−2.0	−1.0	Quiet
	11.30	69	20.5	65	18.5	−3.0	−2.0	"
	12.00	70	21.0	68	20	−2.0	−1.0	Active
	12.30 P.M.	71	21.5	69	20.5	−2.0	−1.0	"
	1.30	70.5	21.3	70	21.0	0.5	0	"
	2.00	70	21.0	70	21.0	0	0	"
	3.00	69	20.5	69.5	20.5	0.5	0	"
	4.00	67	19.5	69	20.5	2.0	1	"
	7.00	67	19.5	69	20.5	2.0	1	"
	8.00	66.5	19.5	68	20.0	1.5	0.5	Quiet

Body Temperature Fluctuations on Cooling.—In these experiments *twelve* specimens of the painted variety ranging in weights from 265 to 712 grams, and ten snappers ranging from 152 to 1725 grams were used. Some of the representatives of each group were subjected to rapid environmental drops while in others the drop was made more slowly. This procedure was followed because in the preliminary observations it was noted that differences in rate of cooling seemed to effect slightly the body temperature fluctuations. In some cases the same individuals were rechecked on different days following the same procedure, and while slight divergences appeared in the records, the same general tendencies were apparent. Illustrating the chief points concerned with the rapid cooling of the painted variety data are given in Table II., which is taken from an experiment performed on Aug. 1, on a medium-sized (360 gms.) turtle, and which is fairly typical of them all.

This particular experiment began at nine o'clock in the morning and extended over five hours, the rapid drop in environmental temperature occurring during the first half hour. Although the

TABLE II.

PAINTED TURTLE, ENVIRONMENTAL AND BODY TEMPERATURES.
RAPID COOLING.

Time.	Room Temp.		Sub. Temp.		Env. Temp. H_2O.		Diff.		Activity.
	F.	C.	F.	C.	F.	C.	F.	C.	
9.00 A.M.	63°	17.5°	63°	17.5°	60°	15.5°	3°	2°	Quite active
9.30	63	17.5	62	16.8	36	3.5	26	13.3	Slow move
10.00	64	18	62	16.8	35	3	25	13	" "
10.30	65	18.5	61.5	16.3	34.5	2.5	27	12.8	" "
11.00	67	19.5	61	16	34	2	27	14.0	Quiet
11.30	69	20.5	58	14.5	34	2	24	12.5	"
12.00	70	21.0	56.6	13.3	33	0.5	23	12.5	"
12.30 P.M.	71	21.5	56.5	13.2	33.5	0.6	23	12.3	"
1.30	70.5	21.3	56	13	33	0.5	23	12.5	"
2.00	70	21	56	13	34 5	2	21.5	12	"

active movements become somewhat subdued when the animal is subjected to a rapid drop in environmental temperature during the first half hour, as indicated in the table, the fact that the body temperature remains at so conspicuously a high level would seem to indicate that the shock of the cold acts as a stimulus and is compensated for, perhaps nervously, by a sudden

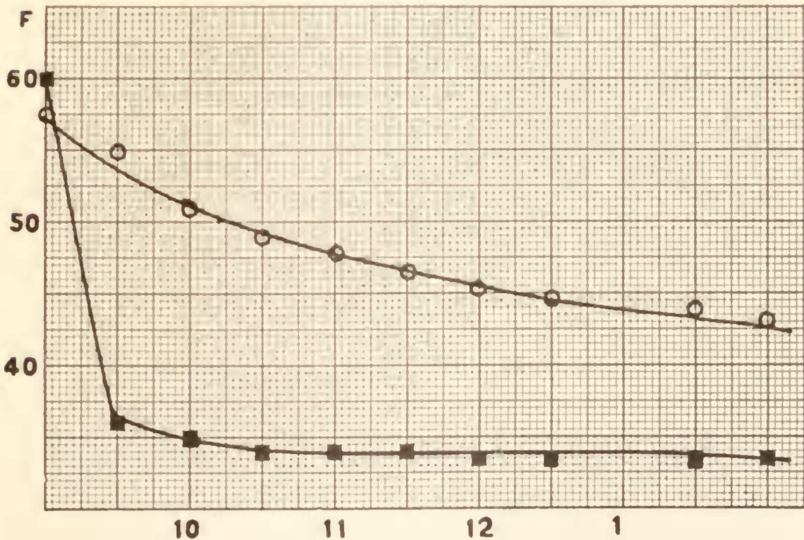

FIG. 2. Plot showing the average body temperature drop from seven painted turtles, correlated with a rapid environmental drop. The body temperatures are represented by circles, the environmental temperatures by squares.

increase in the production of heat. The temperature correlations from seven similar experiments are averaged and plotted against the time intervals and are shown in Fig. 2. Although the body temperature as a rule shows an immediate drop, it is not nearly commensurate with the rapid decline of the surrounding water, and it is conspicuous that during the succeeding four hours the differences are considerable and on the average remain from 8 to 10° F. above that of the environment.

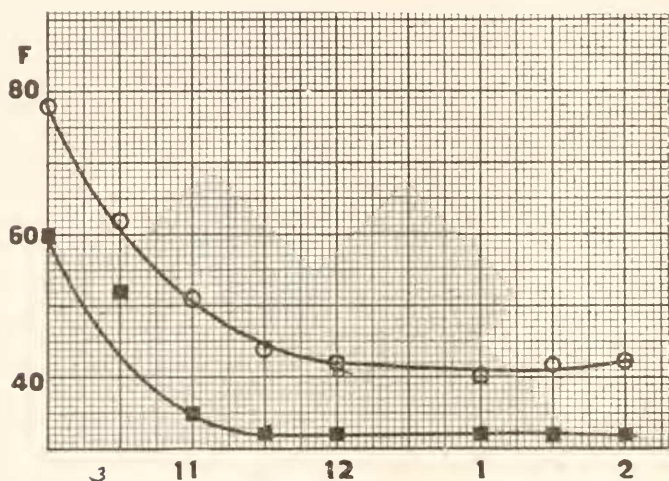

FIG. 3. Plot showing the average body temperature drop from five painted turtles, correlated with a slow environmental drop.

On the following and succeeding days, experiments with the same and other individuals of about equal weight were performed where the animals were subjected to slow cooling which extended over a period of two hours and were then maintained at the temperature of melting ice for another interval. Data from a typical experiment on a painted turtle, are shown in Table III., and the plot of the average fluctuations in temperatures from five records is given in the plot of Fig. 3.

From the table it is noted that at first the animals are quite active but this activity gradually merges into a period of quiescence as the temperature drops to about 45 degrees where, due probably to slight increase in activity, the drop is checked.

In the case of the body temperature changes in the snapping turtles less conspicuous differences are noted. They follow more

TABLE III.

PAINTED TURTLE, ENVIRONMENTAL AND BODY TEMPERATURES; SLOW COOLING.

Time.	Room Temp.		Subj. Temp.		Env. Temp.		Diff.		Activity.
Aug. 2									
8.30	67° F.	19.5° C.	65.5° F.	18.5° C.	62° F.	16.5° C.	3.5° F.	2° C.	Active
9.00	72	22 2	60	15.5	52	11	8	4.5	"
9.30	75	24	55	12.5	47	8.5	8	4	Quiet
10.00	77.5	25.4	54	12	44	6.9	10	6	Slight activity
10.30	80.3	27	49	9.5	43	6	6	3	Quiet
11.00	81	27.5	45	7	36	3	9	4	"
11.30	82	27.9	43	6	33	0.5	10	5.5	Slight activity
12.00	83	28	42	5.5	33	0.5	9	5	Active
12.30	83	28	42	5.5	33	0.5	9	5	"
1.30	83	28	42	5.5	33	0 5	9	5	"

closely the external environmental drop than the painted form when cooled by either procedure. The chief point of interest in the comparisons is the location of the point of check in the drop which is five degrees lower on the average for the snapping turtle. No doubt these differences may be correlated to some extent, with the comparative differences in extent of soft body parts as compared to the harder parts in the two forms, but data on these points are not yet available. A typical protocol of the experiments with the snapping turtles is as follows. Four snappers were placed in containers at 10 o'clock on August 5th, in water drawn from the tap at a temperature of 60 degrees. They were subjected to slow cooling by the addition of ice to the outer container and records taken at intervals for four hours. In Table IV. are given data from specimen *C* of the series, weighing 582 grams and which is typical of them all.

TABLE IV.

SNAPPING TURTLE. ENVIRONMENTAL AND BODY TEMPERATURES.

Time.	Room Temp.		Subj. Temp.		Env. Temp.		Diff.		Activity.
10.00 A.M.	81° F.	27° C.	78° F.	25.7° C.	60° F.	15.5° C.	18° F.	10.2° C.	Active
10.30	82	27.9	62	16.5	52	11	10	5.5	"
11.00	84	29	51	10.5	35	1.5	16	9	Quiet
11.30	85	29.5	44	6.7	32	0.0	12	6.9	"
12.00	86	30.2	42	5.5	32	0.0	10	5.5	Active
1.00	86	30.2	40	4.4	32	0.0	8	4.4	"
1.30	86	30.2	42	5.5	32	0.0	10	5.5	"
2.00	86	30.2	42	5.5	32	0.0	10	5.0	"

A similar series of experiments was performed on four specimens (*C*, *B*, *N* and *O*) on the following day and the average figures of these contribute to the plot, Fig. 4. The body tem-

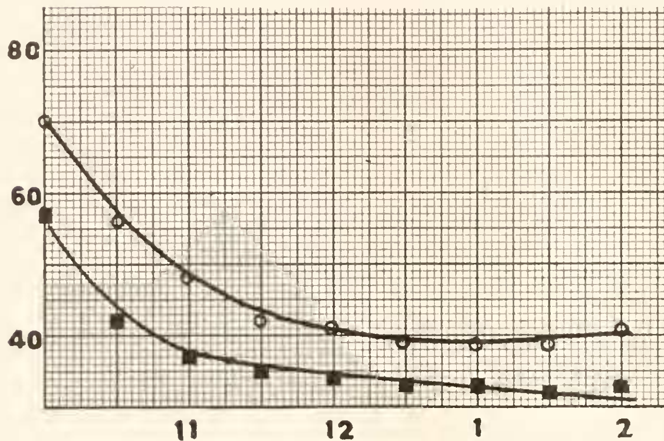

FIG. 4. Plot showing the average body temperature drop from four snapping turtles correlated with a slow environmental drop.

perature curve shows a comparable drop with that of the environment during the first hour and a half but is checked at about 40 degrees.

Body Temperature Fluctuations on Warming.—Two procedures were used in these experiments. On warm days with bright sunlight the animals were placed in containers either in air or in slight amount of water and put directly into the warm rays of the sun. In other cases the temperature of the containers was slowly raised by use of an alcohol lamp through a copper conducting unit. The results seemed not to differ greatly in either procedure. As a rule it was found that the animals could withstand gradual increase in temperature in air better than in water. This was due in part, no doubt, to slight though appreciable transpiration afforded in the former condition. As illustrative of the reactions of the painted variety to gradual increase in temperature, data from specimen *1* are given in Table V. This particular experiment together with others was carried out on an unusually favorable day, August 6. It was noted that hundreds of turtles were basking in the afternoon sun on old logs in the cove north of the laboratory, and it was thought

that observations under experimental conditions might yield interesting although not exactly comparable results. The specimens were taken directly from the lake and fastened in containers in direct sunlight, one thermometer giving body readings and another, in air, the environmental changes. The surface temperature of the lake at this time was 20.5 degrees C. or 69 degrees F., and the initial temperatures of the turtle as they were taken, was only a few degrees less. But at the start of the experiment, after a lapse of some fifteen minutes, due to excitement and activity in the containers, it had approximated the air temperature.

Table V.

Time.	Subj. Temp.		Env. Temp.		Diff.		Activity.
Aug. 6							
1.45 P.M.	77° F.	25° C.	76° F.	24.5° C.	1° F.	0.5° C	Very active
2.00	78	25.7	79	26	1	0.3	" "
2.15	79	26	80	26.8	− 1	−0.8	" "
2.30	92	33.5	106	41.2	−14	−7.7	Gasping, frothing, moisture about eyes. Active.
2.45	102	39	111	44.5	− 9	−5.5	Gasping, frothing, small droplets about eyes.
3.00	99	37.3	109	43	−10	−6.3	Rapid breathing.
3.15	93	34	104	40.1	−11	−6.1	Active, gasping, rapid breathing.
3.30	90.1	32.5	95	35	− 5	−2.3	Restless but frothing
4.00	80	26.8	80	26.8	0	0	Slowly moving

Whether or not the same relative increase in body temperature occurs in the case of the animals observed on the log, is of course, questionable. It was noted that the appendages were outstretched as well as the neck and that after an interval of an hour or more thus exposed, as a rule, a return to the water was made. No doubt also, some slight air currents played upon the body surface, thereby favoring any transpiration that might have occurred. Under experimental conditions, the operation of such factors to help keep the temperature down was very limited.

The average fluctuations in body temperature of five individuals are plotted against increased environmental temperature and shown in Fig. 5. It is noted there are points on the plot of the environmental curve which are somewhat widely displaced.

This is due to the fact that it is difficult to control the environmental temperature throughout the whole period, although in general, the trend of increase and decrease is fairly definite.

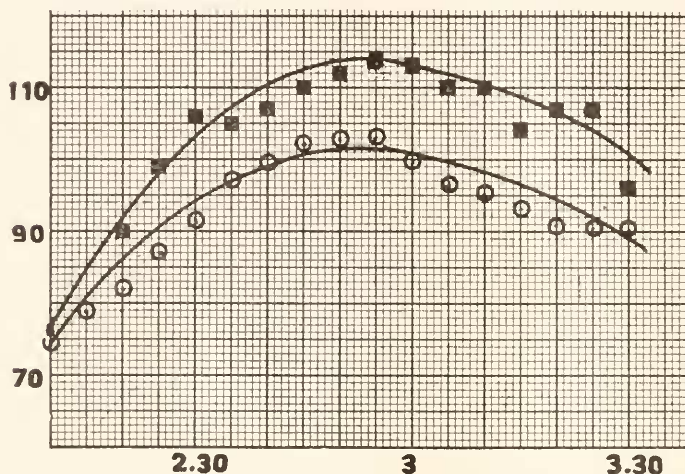

FIG. 5. Plot showing the average body temperature fluctuations of five painted turtles correlated with environmental rise.

The snapping turtle, in experiments under similar conditions, seems to indicate the same general trend, although these animals apparently do not increase in body temperature as rapidly, especially during the initial rise of environmental temperature as does the painted form. This slight difference, however, is transient if the increase in environmental temperature is steady, and eventually at the high critical points the body temperature as well as the reactions in this form simulate those in the other form. Data averaged and plotted from four individuals and checked against the environmental rise are shown in Fig. 6. It is noted on comparison with the preceding plot that increments of environmental temperature are not quite as effective in raising the body temperature in the snappers as in the painted variety. On anatomical grounds one is tempted to attribute these differences in part to a greater radiating surface of soft parts exposed in the snappers, for it is well known that the relative extent of the plastron in this form is considerably less than it is in the painted form. Whether there are in addition, physiological differences in the two forms can not at this time

be stated on the basis of these experiments alone. The comparative behavior of the two forms taken as criteria, would certainly suggest that there might be. Thus the typical signs of discomfort, with rapid respiration and frothing about the mouth and the accumulation of moisture around the eyes appear

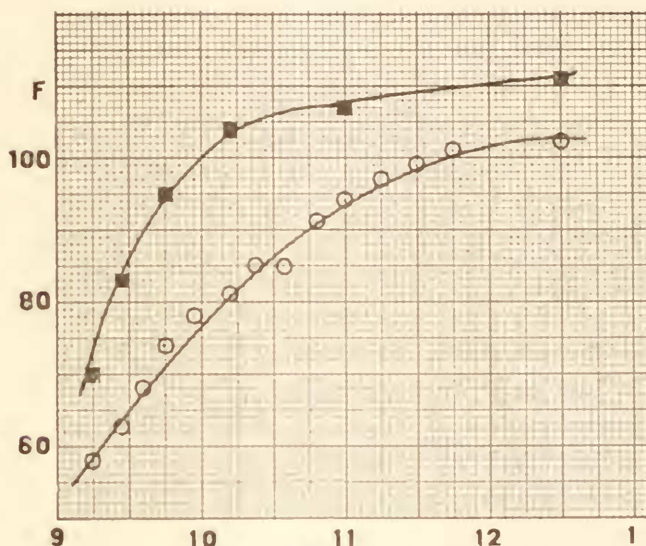

FIG. 6. Plot showing the average body temperature fluctuation of four snapping turtles correlated with environmental rise.

at a body temperature, some ten degrees higher in the snapper than is the case with the painted turtle. At the high critical temperatures however, little differences in endurance could be noted in the two forms. From the limited data accumulated on this point, it appears that neither form withstands a body temperature maintained at between 102 and 105 degrees F. (39–41° C.) for thirty minutes or longer, and in the majority of individuals death results in a much less time.

When placed in water as the surrounding medium and subjected to gradual increase in temperature animals of both groups are apparently incapacitated in their resistance. A typical experiment is cited in Table VI. This animal, a painted turtle, weighing 520 grams, was placed in a container of water at 9.30 on August 2. The water was drawn from the laboratory tap at practically room temperature and was heated during the

extent of the experiment by a small alcohol flame through conduction. It is noted that the body temperature lagged slightly but kept pace with the environmental rise. At the end of two and one half hours, the temperature had reached the critical point and the animal was removed in a moribund condition from which it did not recover. Several other experiments with other individuals eventuated similarly.

TABLE VI.

Time.	Room Temp.		Subj. Temp.		Envir. Temp. (H_2O).		Diff.		Activity.
9.30	75° F.	23° C.	69.5° F.	21° C.	64° F.	18° C.	5.0° F.	+3.0° C.	Active
10.00	77.5	23.3	79	26.5	81	27.3	−3	− .8	Active
10.30	80.3	27	88	31.2	88	31.2	0	0	Very active
11.00	81	27.3	92	33.5	99	37.5	−7	−4	Scratching. Head outstretched above water.
11.30	82	27.9	104	40	108	42.5	−4	−2	Quiet, dead when removed.

Summary.—1. The rectal fluctuations of the common painted and snapping turtles of various sizes and weights are followed through ranges of non-critical and critical high and low environmental changes. In the non-critical range (50–80° F., 10–27° C.) in both forms the fluctuations are found to vary from 3 to 6 degrees F. (1.5 to 3° C.). When the environmental drop is rapid on cooling the rectal reading shows a somewhat greater lag than when cooled more rapidly. In both procedures a check in drop appears at about 40 degrees F., (4.5° C.) and there is maintained for a considerable interval of time.

2. Accompanying these temperature changes are noted differences in physiological activities, with muscular action at the outset which merges into a period of comparative quiet, this in turn followed by slow continuous movements.

3. Increase in environmental temperature is accompanied by a corresponding rise in body temperature and as a rule this is fatal if maintained at 102 to 105 degrees F., for any considerable

time, (30 minutes or more). At 80 degrees and above, animals show marked increased activity; signs of discomfort with rapid respiration; a frothing about the mouth and an accumulation of moisture upon the head and about the eyes.

4. In the absence of concrete data on comparative metabolic rate at different temperatures, these facts are tentatively interpreted to mean that there is in turtles a slight tendency to compensate for critical temperature changes in their environment.

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ON THE COLOR CHANGES IN THE SKIN OF THE LIZARD *PTYCHOZOÖN HOMALOCEPHALUM.*

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The javanese lizard *Ptychozoön homalocephalum* Crvdt. can change its color in some degree in connection with the difference in the color of its surroundings. In bright sunlight the dorsal surface of the animal is light gray with the exception of a number of pronounced blackish brown stripes which have a zigzag course in transversal direction and some spots of the same color at the lateral parts of its head and neck. In strongly shady surroundings the color of the skin between the transversal stripes darkens and assumes almost the same dark color as the stripes, which thereby become almost invisible. With these animals, which very commonly occur in 's Lands Plantentuin at Buitenzorg I have made some experiments to make out whether the change of color is controlled by the vision of the animals or if it is only due to the influence of light acting on the skin directly.

The experiments were made in August 1921 in the Treub-laboratorium at Buitenzorg with animals captured in the Plantentuin, in most cases by natives. I kept the animals in glass vessels with a diameter of about 20 cm., the bottom and sides of which were covered by white paper or black velvet. In these vessels they behaved quite calmly. The experiments consisted in the comparison of the color of the skin of the same animal (1) in a white-covered vessel, (2) in a black-covered vessel, and (3) in a white-covered vessel whilst its eyes were covered with a not transparent cap. The latter consisted of the cut-off digit of a rubber glove, in the top of which a small hole was cut. This cap was pushed over the head of the animal. It covered its eyes and the greater part of its head and neck, whilst the end of the snout was free and the respiration was not hindered. When the animal had been in the same conditions for some time (at least a quarter of an hour) I noted the color

of the skin of its dorsal surface and then placed it in another vessel under different conditions.

Two of my animals I have photographed under the influences of the above-mentioned different conditions. From each of these six photographs were made on one morning, in the case of one of them in the following order: (1) head free, on white back-

FIG. 1. *Ptychozoon homalocephalum*, on a white background. About $\frac{2}{3}$ nat. size.
FIG. 2. *Ptychozoon homalocephalum*, the animal of Fig. 1, on a white background with covered head. About $\frac{2}{3}$ nat. size.

ground, (2) head covered with cap, on white background, (3) head free, on black background, (4) head covered with cap, on white background, (5) head free, on black background, (6) head free, on white background. In a second series of photographs, taken from the other lizard, the succession of experiments was different, but with each exposure the conditions were different from those before and twice the animal was photographed under the same conditions. The photographs taken from animals in

the same conditions always gave the same results of the coloration of the skin.

As is shown by Fig. 1 the transversal stripes are very clearly visible when the animal with uncovered head is on a white background. The parts of the skin between these stripes have a light gray color which is brightest immediately behind the stripes

Fig. 3. *Ptychozoön homalocephalum*, another specimen than that of Figs. 1 and 2, on a black background. About $\frac{2}{3}$ nat. size.

and somewhat darker before them. A sharp contrast with this figure is that of Fig. 2. Here the animal is photographed on a white background, but with covered head. Now the color of the skin is almost uniformly dark gray whilst the transversal dark stripes are more indistinct. Almost the same color is assumed by *Ptychozoön* when it is surrounded by black (Fig. 3). Then also the stripes are only faint and the whole surface is almost uniformly dark gray.

I have made no experiments as to the length of time these lizards require for a complete change of color. Anyhow it takes less than a quarter of an hour. The photographs have been made with Ilford Panchromatic Plates, which were each exposed during one and a half minutes. The animals remained during this comparatively long time almost immobile on the same spot. The plates are developed in the same liquid during quite the same time. They have not at all been retouched or altered in any way. The positives are made with gaslight paper, those of Figs. 1 and 2 are exposed for quite the same time and have afterwards been worked out into a cliché together. Therefore these figures give reliable data for a comparison of the color of the skin of the same animal on a white background with free and with covered eyes. The technical peculiarities given above are mentioned to prove that the two figures give accurate data for the differences of the color under these different circumstances. Fig. 3 can only less directly be compared with the others, but it shows clearly enough that on a black background the color of the skin is of an almost even shade. The plate of this figure has been exposed for the same time as those of Figs. 1 and 2, but owing to the black background it is more or less underexposed. Therefore the animal appears brighter than it was in reality.

The external influences in these experiments were almost quite constant. I studied the color changes of *Ptychozoön* always on quite the same spot in diffuse daylight at a distance of circa 1 m. from the window. The *intensity of the light* was always practically the same, the time being the dry monsoon, when between 8 and 12 A.M. the intensity of the light in the tropics is quite equal. The difference in maximum and minimum of the *temperature* (if there was any difference at all) was very slight and also the *degree of humidity* did not vary noticeably during the experiments. The *structure of the substratum* was in all experiments the same. This, however, proved to be of little importance as was shown by a little minor experiment. Once I tried the same experiments by bringing the animals successively on white paper and on black velvet, but the color changes were the same as in the experiments with the animals in differently clad glass-vessels.

From the above stated peculiarities we may conclude that

Ptychozoön, when the external conditions are quite the same except the color of its surroundings, assumes the color which simulates in the highest degree that which it sees with its eyes. In other reptiles no instances are known that the changes of color are influenced by the vision of the animals, and also experiments on this question only gave negative results (cp. van Rynberk, 1906; Fuchs, 1914).

An important fact is moreover that the color of the skin of *Ptychozoön*, when the temperature is constant, becomes lighter on a white background and darkens on a black one. After Parker (1906) the pigment-cells of all reptiles expand in the light, whilst they contract in the darkness. In those cases, where by other authors different conclusions have been made, these would be due to changes of temperature which had not been taken into account by these authors. In those experiments in which the color changes of reptiles alternately in the shade and in the sunlight were studied, heat reactions may have influenced the movements of the chromatophores, in my experiments, however, the animals remained always on the same spot in diffuse daylight. The temperature was always very uniform and the lizards reacted directly on stimuli of white and black surroundings in the above described manner.

A contradiction to Parker's theory is found in the statements given by Thilenius (1897) for *Varanus*. According to this author *Varanus* assumes a dark color in the shade in 45–50°, whilst in the sunlight at a temperature of less than 30° the color becomes light. Parker has expressed his doubts as to the correctness of these temperatures, which might have been read from an ordinary mercury-bulb thermometer. With a more precise instrument the result would have been a much higher temperature. The difference between the true temperature and the one recorded in this case, however, would then have been more than 20°, and this difference is too large to be put on account of the inaccuracy of the instruments. In the case of *Ptychozoön* the color changes take place in the same way as described by Thilenius for *Varanus* and this proves that the chromatophores of at least some lizards contract in light and expand in dark.

In another way Fuchs (1914) has tried to explain the fact that light in some reptiles causes an expansion and in others a con-

traction of the chromatophores. Firstly he points to the fact that larvæ of amphibians show a different reaction to light in the different stages of development. In *Amblystoma* Babàk (1910) found that the very young larvæ react clearly on stimuli of light, becoming dark in the light and light in the shade. The eyes then have as yet no influence on the color changes. Afterwards, when the larvæ have grown older, the light has a completely other effect on the larvæ: the older larvæ become light in the light and assume a dark color in the dark. This reaction takes place under the influence of the eyes.

According to Fuchs the parietal organ, which in young larvæ is well-developed, has the function to impede the contraction of the chromatophores. Afterwards the eyes obtain an influence on the function of the pigment-cells, the illumination of the retina causes then a contraction of the chromatophores. Stimulation of the eyes therefore causes in older larvæ a reaction opposed to that caused by stimulation of the parietal eye. The older the larvæ, the stronger becomes the influence of the eyes as compared with that of the parietal eye. Consequently these animals become dark on a dark background and light on a light one.

In comparison with these different reactions towards light in young and older larvæ of amphibians Fuchs has put forward the hypothesis that in those reptiles, which show an expansion of pigment in light, the impeding influence of the functioning parietal organ is present. In those reptiles which show a contraction of pigment in light according to Fuchs either the parietal organ has lost its function in the course of phylogeny or ontogeny, or the eyes have acquired, as in older larvæ of amphibians, a regulating influence on the reactions towards light, on account of which the original reaction (expansion) was changed into the opposite (retraction).

In *Ptychozoön* no parietal eye is present. The general shape of the organs in this region of the brain is shown in Fig. 4. The epiphysis consists of a closed pouch which by means of a solid trace of cells is connected to the roof of the diencephalon just behind the commissura habenularis superior. It is directed backwards and covered by a protusion of the roof of the diencephalon, the "Zirbelpolster" of German authors. The well-

developed paraphysis is found immediately before the latter. Almost quite the same arrangement and structure of these organs is found in *Platydactylus muralis* L., an allied species, in which the anatomy and development of the parietal organ is described

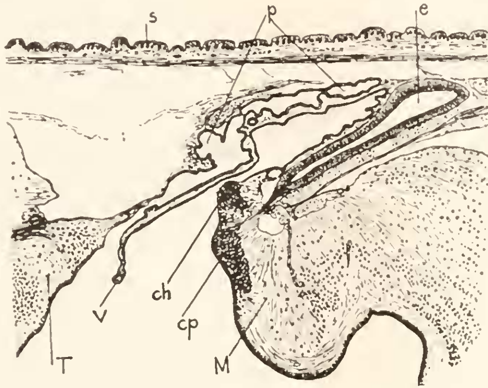

Fig. 4. *Ptychozoön homalocephalum*, newly-hatched specimen. Longitudinal section of a part of the brain. Hæmatoxylin-Delafield, eosin. × 43. *ch*, commissura habenularis superior; *cp*, commissura posterior; *e*, epiphysis cerebri; *M*, mesencephalon; *p*, paraphysis; *S*, skin; *T*, telencephalon; *v*, velum transversum.

at length by Melchers (1900). Besides that from the newly-hatched *Ptychozoön* (with a head-length of 11 mm.) a section of which is shown in the figure, another series of longitudinal sections was made from a younger stage, measuring 9 mm. from snout to occiput. The conditions found here make it highly probable that the development of these organs in *Ptychozoön* is quite the same as that in *Platydactylus muralis*.

In the case of *Ptychozoön* the above-cited hypothesis put forward by Fuchs agrees fairly well with the facts. The data available in the literature, however, are often in contradiction with this hypothesis. In *Platydactylus mauretanicus* the parietal organ is absent and yet this lizard assumes a dark color in the light and becomes light-colored in the dark. On the contrary *Stellio caucasicus* has a well-developed parietal eye and notwithstanding that the animal becomes light-colored in the light and dark in dark surroundings (cp. Studniczka, 1905, and Fuchs, 1914). These two instances already prove that there is insufficient evidence to uphold the above-mentioned hypothesis of Fuchs.

The microscopical structure of the skin in the lighter parts of the dorsal region of *Ptychozoön* differs in some respects with that in the dark stripes. In the light-gray parts (Fig. 5) the epidermis

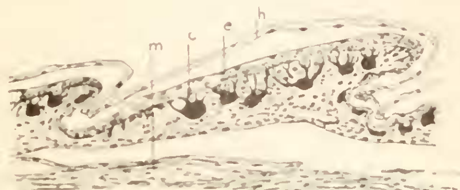

FIG. 5. *Ptychozoön homalocephalum*, newly-hatched specimen. Longitudinal section of a part of a light-colored portion of the dorsal region. Hæmatoxylin-Delafield, eosin. × 100. *c*, cutis with chromatophores; *e*, cell-layer of epidermis; *h*, horn-layer of epidermis, which has loosened from the parts underneath it; *m*, muscles.

contains no pigment at all, the large pigment-cells consist of a large body which is situated in the cutis and a number of ramifications which are directed towards the epidermis and terminate immediately underneath the latter. In the living state a nearly continuous layer of black pigment is distributed under the epidermis by the contraction of the chromatophores. When the pigment-cells expand again, the pigment flows back to the deeper layer of the cutis and the color of the animal becomes much lighter.

In the dark stripes (Fig. 6) the chromatophores of the cutis are usually of a somewhat smaller size than those of the lighter parts of the body, the whole amount of cutis-pigment in a certain

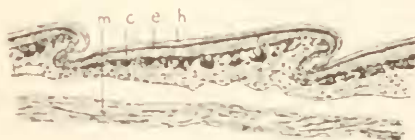

FIG. 6. *Ptychozoön homalocephalum*. Longitudinal section of a part of one of the dark stripes of the dorsal region of the specimen of Fig. 5. Hæmatoxylin-Delafield, eosin. × 100. *c*, cutis with chromatophores; *e*, cell-layer of epidermis, with epidermis-pigment; *h*, horn-layer of epidermis; *m*, muscles.

region, however, is about the same. Besides the cutis-pigment an epidermis-pigment is also present here. The uppermost parts of the cells of the epidermis are crowded with small black pigment granules which are found immediately underneath the

horn-layer of the epidermis. When the chromatophores of the cutis contract, the pigment of the epidermis remains on the same spot and the color of this region retains its black shade. It is only covered by a thin horn-layer and it is therefore much more conspicuous than the pigment of the chromatophores of the cutis, which is covered by the whole ectodermal layer.

The useful effect of these color changes in *Ptychozoön* is a matter of course. The lizards live on the trunk and larger branches of the trees. When they are in shady places they are hidden by their almost uniformly dark color. In the sunlight their light-gray hue equals that of the mossy background. They become still more inconspicuous by the possession of the queer zigzag dark stripes which procures them almost the same color and design as the bark of a tree overgrown with lichens.

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TOXICITY OF OXYGEN FOR PROTOZOA IN VIVO AND IN VITRO: ANIMALS DEFAUNATED WITHOUT INJURY.

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INTRODUCTION.

The fact that oxygen in an excessive amount is toxic for many if not all forms of life was first demonstrated by the numerous and very thorough experiments of Paul Bert ('74). But the swim bladders of some fishes normally contain oxygen at a pressure of 100 atmospheres (Haldane, '22). The cells lining the bladder apparently are acclimatized to the oxygen. On the other hand, Pütter ('05), working on the respiration of protozoa, states that parasitic forms, such, for instance, as *Opalina*, lived many days in a medium from which practically all the free (gaseous) oxygen had been removed. In view of the work of Pütter and that quoted by Haldane it occurred to me that a study of the toxicity of oxygen for the parasitic protozoa of many animals would be a worthy undertaking.

Wood-eating termites have the most abundant and the most varied entozoan fauna of all animals that have been studied.

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Approximately half the body weight of every worker and nymph, except for a few days after molting, is composed of many kinds of large, intestinal flagellates. Recently molted termites are lacking in pigment and for this reason may easily be distinguished from other individuals. There is no difficulty whatever, then, in being absolutely certain that all animals used in experiments harbor a teeming menagerie of protozoa; consequently termites lend themselves most admirably to many kinds of precise experimentation.

In a previous paper the writer ('25*b*) showed that *Trichomonas termopsidis* was entirely removed from the large Pacific Coast termite *Termopsis nevadensis* Hagen after 24 hours' oxygenation at one atmosphere pressure, and that all the other protozoa (*Trichonympha campanula*, *Leidyopsis sphaerica*, *Streblomastix strix*) were removed after 72 hours' oxygenation. The termites suffered no ill-effects *per se* from the oxygenation, although they died within three to four weeks after their protozoa had been removed. Recently this work has been carried further: four widely separated species of termites from two families have been used; and various intervals and amounts of pressure have been employed.

The bearing which these experiments have on the symbiosis between these termites and their intestinal flagellates will be reserved for a later paper. It is sufficient at present to say that the ability of these termites to live on their normal diet of wood is lost after their protozoa have been removed, regardless of the method employed in removing them.

Many other protozoa-harboring animals, such as cockroaches, earthworms, frogs and rats have been oxygenated. Some of the protozoa of the frog, rat, and man have been grown in cultures which have been oxygenated. And oxygenation experiments have been carried out on several free-living protozoa.

Material.

Of the termite family Rhinotermitidae, *Leucotermes tenuis* Hagen from British Guiana and *Reticulitermes flavipes* Kollar from Maryland were used; in the family Kalotermitidae, *Cryptotermes* sp. from British Guiana and *Termopsis nevadensis* Hagen from Oregon were used. Many thanks are due Dr. Alfred Emerson for *Cryptotermes* and *Leucotermes*.

Cockroaches (*Periplaneta americana?*) were obtained in Baltimore. So were all the parasitic protozoa in culture and most of the free-living protozoa. The exact source of the frogs (*Rana pipiens*), fish, salamanders and rats is not known.

Methods.

The protozoa-harboring animals and the protozoan cultures were placed in flasks into which tank oxygen of 97 per cent. purity was run until the air was removed, or practically so. The flasks were then clamped down securely and from 1 (760 mm. mercury) to 2.5 standard atmospheres (except for temperature correction) were added to the 1 atmosphere at the time of the experiment. Other details of methods are given under the experiments on the different animals.

Experiments.

1. *Termites.*

The effect of oxygenation at various pressures on the protozoa of four genera of termites is given in table I. The minimum time

Table I.

Time Required at Various Pressures of Oxygen.

Pressure in atmospheres	To kill all intestinal protozoa of										To Kill *Trichomonas* in Culture from			To Kill Host		
	Termites								Cockroach.	Frog.	Frog.	Rat.	Man.	Termopsis.	Cockroach.	Frog.
	Rhinotermitidæ.				Kalotermitidæ.											
	Leucotermes.		Reticulitermes.		Termopsis.		Cryptotermes.									
	Hrs.	Min.	Hrs.	Min.	Hrs.	Min.	Hrs.	Min.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.
1 . . .	24		*		72		*									
1.5 .	4	30	9		9		7	30								
2 . . .	1	35	4		5		4	30								
2.5 .	1	15	1	40	2		1	55								
3 . . .		50		50	1	5	1									
3.5 .		30		30		40		35	3½	28	6	10	11	45	90	65

* Not killed in ten days.

required to kill the protozoa of termites and other animals was obtained as follows: Several experiments were started at the same time so that any one could be stopped without interfering with

the others, and the experiments were examined one by one until the minimum was found to be within certain limits. Then the experiments were set up again and were examined at various intervals within the known limit. Sometimes it was necessary to repeat the process many times, before a fairly accurate minimum was finally determined. The minimum thus determined was then tested out three times.

Termites were usually examined immediately after having been removed from oxygen and non-motility was the criterion for determining whether or not their protozoa had been killed. It was found that if a few protozoa were motile at the end of the oxygenation period they did not die later. Some hosts which had been freed of motile flagellates were examined at intervals up to ten days, and no protozoa ever appeared in any of them. It is of interest to note that the protozoa disappeared from the intestine very soon—usually within two to four hours—after they had been killed. They were probably digested by the termites.

At one atmosphere the protozoa of *Cryptotermes* and *Reticulitermes* were not all killed in all hosts in ten days; however, they were all killed in a few hosts even in three days. They were all killed in a great majority of hosts in ten days, but in a small number—perhaps about 5 per cent.—some protozoa were alive at the end of ten days.

The protozoa of *Leucotermes* were killed very much more quickly than those of the other termites until a pressure of 2.5 atmospheres was reached. These differences in oxygen toxicity are not correlated with size of termite hosts, for *Termopsis* is approximately twenty times as large as *Leucotermes* and ten times as large as *Cryptotermes*, but *Reticulitermes* and *Leucotermes* are about the same size. Difference in habit may be a factor, but *Reticulitermes* and *Leucotermes* are very similar in habit as well as in structure. The protozoa of these four termites, although all flagellates, are nevertheless quite distinct morphologically, many of them belonging to separate families. Hence, it is possible that the differences in oxygen toxicity may be found to be in the protozoa themselves.

In *Termopsis*, as was true in previous experiments (Cleveland, '25*b*), *Trichomonas* was killed first and *Trichonympha* last until a pressure of three atmospheres was reached. Then a peculiarly

interesting result occurred, *Streblomastix* was killed before *Trichomonas* in many hosts. At a pressure of 2.5 atmospheres, *Trichomonas* and *Streblomastix* are both killed at or about the same time. Regardless of the amount of pressure, the largest protozoa of *Termopsis*, with the exception of one very small form (*Tritrypanoplasma*) not present in all hosts and abundant in only a few, are invariably the last ones to be killed, but in *Cryptotermes* and *Leucotermes* this is not always the case. In *Cryptotermes*, for instance, sometimes all individuals of the largest genus (*Diplonympha?)* are killed when many *Decescovina* are left alive.

Fifty individuals of *Termopsis* were confined in oxygen at 3.5 atmospheres. At the end of forty hours, every individual had become immobile. The experiment was stopped after forty-five hours, when it appeared that every individual was dead, although a few hours later two individuals became feebly motile, and were as active as ever two days later. Forty-five hours at 3.5 atmospheres must be about the time required to kill this termite, which is 67.5 times as long as it takes to kill its protozoa. In other words, oxygen at this pressure is 67.5 times as toxic for the protozoa of termites as for the termites themselves.

The time required to kill the protozoa of these termites at 3.5 atmospheres of oxygen certainly does not injure the termites in the least. Oxygenation at this pressure is surely a very rapid means of freeing termites of their protozoa. It is very much more satisfactory in many ways than incubation (Cleveland, '24), and furnishes a very ready means of determining whether or not all protozoa-harboring termites are dependent on their protozoa to digest their food for them.

In order to determine what effect, if any, partial pressures of other gases, particularly nitrogen, had on oxygen toxicity, termites of each of the four genera that were oxygenated were confined in five atmospheres of air (the partial oxygen pressure of five atmospheres of air approximates the total oxygen pressure of one atmosphere of oxygen) for the same time that they were confined in one atmosphere of oxygen. Five atmospheres of air, in every instance, gave exactly the same result as one atmosphere of oxygen. Thus, the toxicity of oxygen is unaffected by the presence of nitrogen and the rare gases of the air. In another experiment 3.5 atmospheres of air were used with the result that

the protozoa were not killed at all. It is evident, then, that mere mechanical pressure does not kill the protozoa.

2. *Cockroaches.*

Since the protozoa of termites were so easily removed by oxygenation, it immediately became desirable to try the method on other protozoa-harboring insects. The cockroach has many protozoa and can be obtained easily in quantity. By pressing on the abdomen with the fingers or some mechanical instrument and forcing out some of the intestinal contents it is not difficult to determine just what protozoa an individual harbors, and the procedure does not injure the insect. Cockroaches with two ciliates, *Nyctotherus* and *Balantidium*, and two flagellates, *Lophomonas* and *Polymastix*, were oxygenated at 3.5 atmospheres. About 200 insects were used in these experiments. The minimum time required to kill all individuals of each of the four protozoan genera is given in Table II. It is interesting to note that the flagellates were both killed in 40 minutes, the same time required to kill the flagellates of the large Pacific Coast termite (*Termopsis*), at this pressure, while the ciliates were not all killed until $3\frac{1}{2}$ hours, more than five times the time required to kill the flagellates living under identical conditions. From this it would appear that oxygen is actually more toxic for flagellate protozoa.

TABLE II.

TIME REQUIRED AT 3.5 ATMOSPHERES OF OXYGEN TO KILL ALL INDIVIDUALS OF CERTAIN INTESTINAL PROTOZOA OF

Frogs				Cockroaches		
No. of frogs used	Protozoa	Range in hours	Mean in hours	Protozoa	Hrs.	Min.
15	*Hexamitus*	3–7	5	*Lophomonas*		40
10	*Polymastix*	5–11	7	*Polymastix*		40
35	*Trichomonas*	8–15	12	*Nyctotherus*	3	30
30	*Opalina*	12–20	18	*Balantidium*	3	30
3	*Nyctotherus*	28	28			

Two other protozoa, an unidentified flagellate and *Endamœba blattæ*, were present in some of the cockroaches, but not in a sufficient number to make it feasible to work out the minimum time required to kill them. They were killed.

When cockroaches are confined in oxygen at 3.5 atmospheres, they are able to live approximately 90 hours, which is 26 times as long as their ciliate and 135 times as long as their flagellate protozoa live at this pressure. Here, again, as in termites, it is evident that the oxygenation which removes the protozoa of cockroaches certainly does little, if any, harm to the cockroaches themselves. They, unlike termites, live normally (indefinitely) after their protozoa have been removed. Oxygen, then, at 3.5 atmospheres is 135 as toxic for the flagellates and 26 times as toxic for the ciliates living in cockroaches as it is for the insects themselves.

3. *Earthworms.*

Many earthworms, harboring a fairly large number of ciliates of the genus *Hoplitophrya*, were oxygenated at 3.5 atmospheres, but the minimum time required to kill their protozoa was not determined. It is more than six and less than twenty hours.

4. *Frogs.*

After all protozoa had been removed from three invertebrates, it seemed highly desirable next to oxygenate a cold-blooded vertebrate harboring many protozoa. For this work the frog (*Rana pipiens*) was selected.

Most frogs harbor an abundant protozoan fauna; two ciliates, *Opalina* and *Nyctotherus*, and four flagellates, *Trichomonas* (*Tritrichomonas* in the opinion of some investigators), *Chilomastix*, *Hexamitus*, and *Polymastix*, are usually present in a fairly large number of hosts; in fact, all are sometimes present in the same host. Two hundred frogs were procured and just before being used in experiments each individual was examined in order to ascertain what protozoa were present. This examination was made by attaching a No. 7 hard rubber catheter, cut off at the insertion end to four inches in length, to a 5 cc. Luer syringe; by inserting the catheter into the rectum it was possible to draw out all or any amount of the rectal contents, which, when examined under the microscope, revealed immediately what protozoa each frog harbored. Of course, the number of protozoa present was also ascertained at the same time.

When frogs were oxygenated at 3.5 atmospheres, it was found that some of their intestinal protozoa were killed more quickly

than others, so it became necessary to determine the minimum time required to kill all individuals of each protozoan genus, and in working this out it was noticed that the protozoa of a certain genus would be killed more quickly in one host than in another; consequently, in most instances, a fairly large number of hosts was used. The number of hosts used, the range in time and the mean in time required to kill the protozoa, are given in Table II. It has been impossible to think of a plausible explanation of why the protozoa of one frog are affected more adversely by oxygen than those of another. This was noticed when several frogs were oxygenated in the same flask at the same time. The same phenomenon was met in the oxygenation of termites and cockroaches. Perhaps more work will throw light on it.

It is interesting to note that it takes 28 hours to kill the ciliate *Nyctotherus* in the frog and 3½ hours to kill *Nyctotherus* in the cockroach. The flagellate *Polymastix* is killed in 40 minutes in the cockroach while the species of this genus that lives in the frog is not killed until approximately 7 hours. It would be most interesting, indeed, to cultivate *Nyctotherus* and *Polymastix* from both hosts and then subject them to the same oxygen pressure. It is very probable, though not certain, in view of the oxygenation studies of the frog *Trichomonas* in vivo and in vitro, that oxygen is actually more toxic for *Polymastix* and *Nyctotherus* in the cockroach.

Some frogs live as long as 65 hours in 3.5 atmospheres of oxygen, more than twice as long as their ciliate and five to six times as long as their flagellate protozoa.

Twenty tadpoles,[1] 2 with *Nyctotherus*, 3 with *Trichomonas*, 3 with *Opalina*, 4 with *Hexamitus*, and 8 with *Euglenamorpha*, were oxygenated at 3.5 atmospheres with the result that their protozoa were killed in approximately the same time as those of frogs. *Euglenamorpha* is not present in adult frogs, and it was primarily for this reason that tadpoles were oxygenated. This flagellate is very similar morphologically to plant-like free-living protozoa of the genus *Euglena* which, as will be seen later, must be oxygenated sixty five hours at 3.5 atmospheres before being killed, while

[1] The method used to determine what protozoa each tadpole harbored was simple: Each individual was placed to itself in a small vesel; very soon a considerable quantity of fecal material was passed, which, when macerated and examined under the microscope, revealed the protozoa harbored.

Euglenamorpha is killed in approximately thirty hours. It is impossible to say whether this difference in oxygen toxicity is due to environment or metabolism. Here is an interesting situation, calling for careful investigation.

5. *Goldfish and Salamanders.*

After having removed all protozoa from an air breathing cold-blooded vertebrate, the frog, it seemed expedient to oxygenate a water breathing vertebrate harboring many protozoa. Goldfish and salamanders (*Necturus*) were used for this purpose. Twenty young goldfish, harboring large numbers of intestinal flagellates of the genus *Hexamitus*, and ten young salamanders, harboring large numbers of intestinal flagellates of the genera *Trichomonas* and *Prowazekella*, were oxygenated at 3.5 atmospheres. The same thing occurred here as in the oxygenation of frogs, namely, some hosts lost their protozoa sooner than others; *Hexamitus* in some goldfish was killed in 4 hours and in others not until 5 hours; *Trichomonas* and *Prowazekella* in some salamanders were killed in 9 to 10 hours and in others not until 11 to 12 hours. In adult hosts it would probably take slightly longer to kill all protozoa in all hosts. In the material used in this study all individuals of *Trichomonas* and *Prowazekella* were killed in all hosts in 12 hours and all individuals of *Hexamitus* in all hosts in 5 hours, while the hosts were not killed before 50 to 60 hours.

It is interesting to note how closely these death points of *Hexamitus* and *Trichomonas* inhabiting the water breathing vertebrates, salamanders and goldfish, parallel those of the *Hexamitus* and *Trichomonas* that inhabit the air breathing vertebrate, the frog (see Table II).

It is quite probable that the external parasitic ciliates of fish would be killed by oxygenation and without injury to the fish. These and other protozoan parasites, according to reports, do considerable damage to fish, which oxygenation would probably check.

What effect oxygenation would have on the hundreds of species of sporozoa in fishes should be determined.

6. *Rats.*

The next logical step in the development of the work was to oxygenate a warm-blooded vertebrate. *Trichomonas* (*Tritrichomonas* in the opinion of some investigators) is present in a fairly large number of rats; because of this, and owing to the ease with which rats may be obtained, the rat was selected. In order to demonstrate the presence of protozoa, fecal contents were removed by means of a catheter, as in the experiments with frogs, and were examined under the microscope.

It was found, however, that the rats themselves were not able to live more than five to six hours at 3.5 atmospheres of oxygen and that their protozoa were not killed in this time.

7. *Trichomonas from Frog, Rat and Man in Culture.*

Since it was impossible to remove the protozoa from a warm-blooded vertebrate by the method employed in removing them from the cold-blooded vertebrate, the problem of the toxicity of oxygen for the protozoa living in the intestines of rat and man was attacked in another manner, viz. the protozoa were grown in culture [1] and the cultures were oxygenated at 3.5 atmospheres by placing a few drops of the fluid from each culture in the same flasks that had been used in all the other experiments.

It was found (see Table I.) that the *Trichomonas* of frogs was killed in six hours, the *Trichomonas* of rats in ten hours, and the *Trichomonas* of man in eleven hours. Obviously, it is impossible, then, to kill the protozoa of the rat and of man by oxygenation at this pressure without killing the hosts themselves first.

It is interesting to note that the frog *Trichomonas* in culture is killed in about one-half the time required to kill it in the frog. This is perhaps explained in part by two facts: (1) oxygen is more soluble in water than in blood, and (2) the host furnishes some sort of resistance or barrier which makes it slightly more difficult for the oxygen to reach the protozoa.

[1] Several of the culture media that have been employed by other investigators were used, but the following medium, which is largely a compilation from other methods, was found to be very satisfactory. For frog *Trichomonas*, sodium citrate 1 per cent, sodium chloride 0.5 per cent, Löffler's dehydrated beef serum 0.5 gram, distilled water 100 cc.; for *Trichomonas* of man and rat, 0.2 per cent, more NaCl was used. Growth was very abundant. Subcultures were made every three days of the organisms from rat and man. The frog *Trichomonas* lived three months sometimes without being transferred.

These experiments, as well as those on free-living protozoa, show that oxygen is directly toxic for protozoa, that it acts on them directly and not through any tissues of their hosts. In other words, the tissues of the hosts are not stimulated by the oxygen to give off products which kill the protozoa.

8. *Free-living Protozoa.*

It seemed highly desirable to compare the toxicity of oxygen for free-living protozoa with that for parasitic or entozoic protozoa; consequently, several genera of flagellates and ciliates were selected and were oxygenated in the same manner as were the cultures of parasitic protozoa.

TABLE III.

APPROXIMATE MINIMUM TIME REQUIRED AT AN OXYGEN PRESSURE OF 3.5 ATMOSPHERES TO KILL ALL INDIVIDUALS OF CERTAIN FREE-LIVING PROTOZOA.

Ciliates	Hours	Flagellates	Hours
Paramoecium	5	*Euglena*	65
Chilodon	4	*Heteronema*	50
Diophrys	60		
Holostica	50		

The results of these experiments are given in Table III. The fact that *Paramœcium* is killed in 5 hours, *Chilodon* in 4, *Holostica* in 50, and *Diophrys* in 60 shows conclusively that oxygen is just as toxic for some free-living ciliates as it is for some parasitic ciliates and flagellates. It is actually even more toxic for *Paramœcium* and *Chilodon* than for *Trichomonas* of frog, rat, and man in culture; the former are killed in 5 and 4 hours respectively, while it required 6, 10 and 11 hours respectively to kill the latter. And we have seen that the frog *Trichomonas* in culture is killed in about half the time required to kill it in the frog, yet it is killed much sooner than the ciliates, *Opalina* and *Nyctotherus*. It has not been possible to cultivate *Nyctotherus* and *Opalina* from frogs, but, reasoning from the time required to kill *Trichomonas* in vivo and in vitro, it would take 10 to 12 hours to kill them in culture, or more than twice the time required to kill *Paramœcium* and *Chilodon*. I cannot explain why oxygen is less toxic for *Diophrys* and *Holostica* than for *Paramœcium* and *Chilodon*. Perhaps a combined study of the metabolism, habitat, and oxygen toxicity

of these and many other free-living ciliates will throw light on the problem.

After having oxygenated the ciliates, the results with the plant-like flagellates *Euglena* and *Heteronema* are not surprising. Some animal-like free-living flagellates would probably yield toxicity results quite similar to those obtained with parasitic flagellates.

Summary and Conclusions.

The toxicity of oxygen at various pressures for four genera of termites has been determined. At a pressure of 3.5 atmospheres the protozoa are all killed in two genera in 30 minutes, in one in 35 minutes, and in another in 40 minutes, while the termites themselves are not killed until 45 hours. Thus, oxygen is more than forty times as toxic for the protozoa as it is for the termites. This makes it possible to remove all protozoa from termites very easily and without injury to the host.

The protozoa of two termite genera were not killed at one atmosphere of oxygen even in ten days, while in two other genera they were killed in one and three days respectively. This gave an excellent opportunity to work out what effect, if any, partial pressures of other gases of the air, particularly nitrogen, had on oxygen toxicity. All four genera when confined in five atmospheres of air (partial O_2 pressure of 5 atms. of air approximates the total O_2 pressure of 1 atm. of O_2) gave exactly the same result as when confined in one atmosphere of oxygen for the same time. Thus, the toxicity of oxygen is in no way connected with or affected by the partial pressures of other gases of the air. It is the partial pressure of oxygen, and not mere mechanical pressure, that matters.

Cockroaches harbor many kinds of protozoa, all of which were removed by oxygenation at 3.5 atmospheres in $3\frac{1}{2}$ hours; the flagellates, *Lophomonas* and *Polymastix*, were killed in 40 minutes, and the ciliates, *Nyctotherus* and *Balantidium*, in $3\frac{1}{2}$ hours. The cockroaches themselves were not killed until 90 hours. Thus, oxygen at this pressure is 135 times as toxic for the flagellates and 26 times as toxic for the ciliates living in cockroaches as it is for the insects themselves.

It is highly probable that all insect-inhabiting protozoa may be removed by oxygenation without injury to their hosts. If so, the

rôle which insects play in the transmission of protozoa from man to man, from animal to animal, from animal to man and from plant to plant can be worked out much more effectively. What effect, if any, oxygenation would have on other insect-transmitted organisms, bodies, inclusions, and agents would be well worth study.

Earthworms when oxygenated lose their ciliates and are uninjured by the process.

Frogs harbor many protozoa. More than 150 experiments have been carried out on the oxygenation of frogs, and all the intestinal protozoa may be removed without injury to the frogs. Table II shows the minimum time required to kill three flagellates, *Hexamitus*, *Polymastix* and *Trichomonas*, and two ciliates, *Opalina* and *Nyctotherus*. The ciliates are killed in less than one-half the time required to kill the frogs, and the flagellates in one-fifth to one-tenth the time.

The protozoa of two water breathing vertebrates, goldfish and salamanders, were all killed by oxygenation in less than one fifth the time required to kill their hosts.

If oxygenation will remove the protozoa of other amphibia, it will be possible to make some interesting studies on protozoal host specificity.

It is highly probable that all intestinal flagellates and ciliates may be removed from all invertebrates and from all cold-blooded vertebrates by oxygenation and that none of these hosts will be injured. It is also possible that the sporozoa, amœbæ, and blood-inhabiting protozoa may be removed from the same hosts in the same way and without injury to the hosts.

Many experiments have been carried out on *Trichomonas* from frog, rat and man in culture. All of these protozoa are killed by oxygenation (see table I for the minimum time), but the time required to kill them in all except the frog is longer than it takes to kill the host itself at the same pressure; so it is impossible to remove the protozoa from rats and human beings by confining them in oxygen at 3.5 atmospheres. Perhaps oxygen may be successfully administered to warm-blooded vertebrates in some other way. Work of this nature is in progress.

Oxygenation experiments have been carried out on four genera of free-living ciliates and two of free-living flagellates. Oxygen

is certainly just as toxic for some free-living ciliates as it is for parasitic ciliates; for others, it is not. For *Paramœcium* and *Chilodon*, it is really more toxic; for *Diophrys* and *Holostica*, it is considerably less toxic. It is not very toxic for two plant-like flagellates, *Euglena* and *Heteronema*, but would probably be found to be just as toxic for some animal-like free-living flagellates as for some parasitic species.

Oxygen in excessive amounts is toxic for all animals, but protozoa possibly take up a correspondingly larger amount of it as the tension or pressure is increased than do higher animals and for this reason are affected more adversely than termites, cockroaches, earthworms and frogs. During oxygenation the protoplasm of the protozoa sometimes becomes very much vacuolated,[1] which may indicate that it is being consumed, perhaps actually burned up, by increased metabolism. However, the metabolism of higher vertebrates is said to be slowed down by increased oxygen pressure. But Amberson, Mayerson, and Scott ('24) were "able to show that the metabolic rate in some of the higher marine invertebrates, with well developed respiratory mechanisms, is closely dependent upon the oxygen tension in the water over a wide range."

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[1] It is also true that many dying protozoa, regardless of the cause of death, sometimes become vacuolated.

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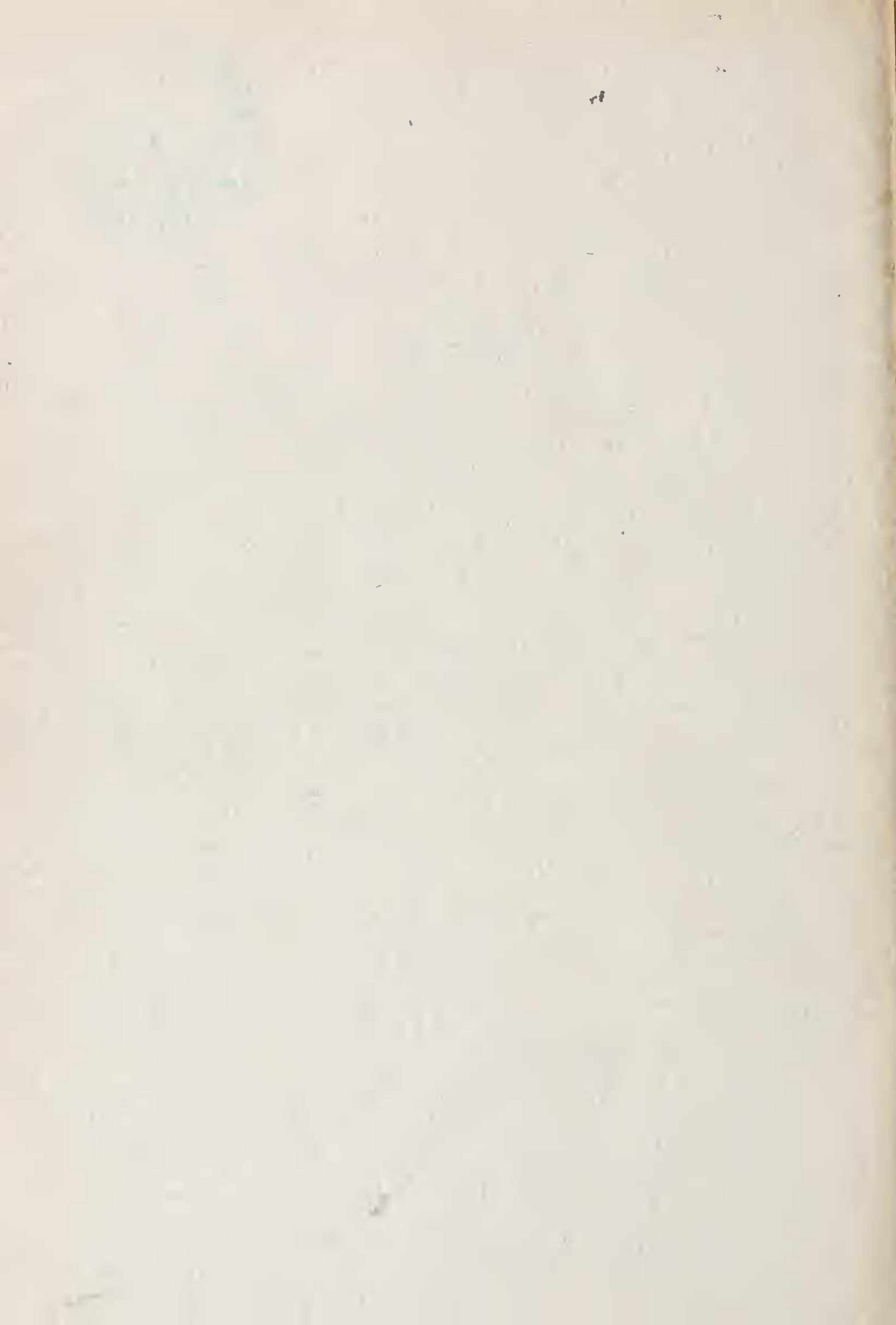

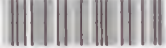
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